PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)
Chandrasekhar Satishchandran *et al.*	) Confirmation Number: 5538
U.S. Patent Application No.: 10/009,134	) Group Art Unit: 1635
Filed: October 20, 2002	) Examiner: Kimberly Chong
For: METHODS AND COMPOSITIONS FOR INHIBITING THE FUNCTION OF POLYNUCLEOTIDE SEQUENCES	)

DECLARATION UNDER 37 C.F.R. §1.132

I, Daniel McCallus, declare that:

1. I currently hold the position of Director, Vector Technology with Nucleonics, Inc. I have a Ph.D in Immunology and have worked in the field of nucleic acid expression systems, including DNA vaccines, since 1993 and in the field of RNA interference as well as other areas of RNA biology from 2002 to the present. A copy of my Curriculum Vitae is submitted herewith as Attachment A.

2. I am familiar with the above-referenced application and its prosecution, including the rejections of the claims as set forth in the Office Action dated September 7, 2007. I am also familiar with Werther *et al.* (U.S. Patent No. 5,929,040), Fire *et al.* (U.S. Patent No. 6,506,559), Heifetz *et al.* (WO 99/61631), Calabretta *et al.* (U.S. Patent no. 5,734,039), and Thompson *et al.* (U.S. Patent No. 6,146,886), all of which have been cited against the current pending claims.

3. At the time the invention was made, it was generally believed that inhibition of gene expression by RNA interference as reported in nematodes would not be a viable mechanism for

sequence-specific inhibition of gene expression in mammalian cells due to the induction of the interferon response (also known as the PKR response). It was well known in the field that long double-stranded RNA molecules in particular activated an interferon-induced protein kinase (PKR, previously known as DAI) in mammalian cells that produced global effects on gene expression and in some cases, induced apoptosis.

4. The well-documented PKR response spurred investigators to examine RNA interference mechanisms in mammalian cells in more detail. It was known that activation of PKR by double stranded RNA molecules was length-dependent, and efficiency in activating the enzyme increased with increasing chain length (see Manche *et al.* (1992) Mol. Cell. Bio. 12: 5238-5248 (Attachment B)). Double stranded RNA molecules less than 30 base pairs failed to activate PKR, while maximum activation appeared to occur with double stranded RNA molecules longer than 80 base pairs (see Minks *et al.* (1979) J. Biol. Chem. 254: 10180-10183 (Attachment C)). Given the extensive literature on the non-specific effects on inhibition of gene expression and induction of the PKR response with long double stranded RNA molecules in mammalian cells, I did not believe prior to the present invention that mammalian genes could be targeted for sequence-specific gene expression with double stranded RNA molecules longer than 30 base pairs. Confirming my notions, several reports were published describing the necessity to limit the length of the double stranded RNA molecules to less than 30 base pairs to avoid the PKR response and achieve sequence-specific gene silencing (see for example Caplen et al. (2001) Proc. Natl. Acad Sci. USA 98: 9742-9747 (Attachment D) and Elbashir et al. (2001) Nature 411: 494–498 (Attachment E)).

5. As a person having much experience and knowledge in this field, I do not believe that the claimed invention would have been obvious in April of 2000 from the disclosures of the references recited in paragraph 2. Specifically, Fire *et al.* and Heifetz *et al.* describe methods of target gene silencing using double stranded RNA molecules in nematodes and plants, respectively. These organisms do not exhibit the PKR response to double stranded RNA molecules as mammals do. For the reasons discussed in paragraphs 3 and 4, a scientist working in this field would not have been motivated to substitute the double stranded RNA molecules disclosed in Fire *et al.* and Heifetz *et al.* for the antisense sequences in the constructs disclosed in

Werther *et al.* and Calabretta *et al.* to silence target gene expression in mammalian cells. It was known that long double stranded RNA molecules produced non-specific inhibition of gene expression in mammalian cells and did not result in sequence-specific gene silencing as observed in other systems. Thus, the skilled artisan would not have expected to achieve sequence-specific gene silencing in mammalian cells with the double stranded RNA molecules used by Fire *et al.* and Heifetz *et al.*, let alone express multitarget double stranded RNA molecules from a single vector as claimed in the present invention.

6. The present invention also provides expression vectors for expressing multiple double stranded RNA molecules from different promoters. In April of 2000, I was aware of the widely reported phenomenon of promoter interference and was concerned that such a construct might not express each of the double-stranded RNA molecules efficiently. Promoter interference is characterized by reduced or complete loss of gene expression from one promoter when multiple transcription units are contained within the same vector. Promoter interference has been observed in various biological systems and has been reported to occur with RNA pol III promoters (see for example Hull et al. (1994) Mol. Cell Bio. 14: 1266-1277 (Attachment F)).

7. The problems encountered with promoter interference when attempting to express two or more genes from a single expression vector prompted alternative approaches, such as inclusion of internal ribosome entry sites in the constructs, to achieve sufficient expression of all the genes. Although this strategy allows one to obtain multiple protein products from a single polycistronic mRNA, it does not allow one to produce multiple RNA products as would be required for the production of antisense or double stranded RNA molecules. Thus, promoter interference remained a recognized problem to the skilled artisan, and although solutions were available if one were interested in obtaining expression of multiple protein products, these solutions were not applicable to the expression of multiple RNAs.

8. Calabretta et al. describe a composition containing two different antisense molecules and speculate that the two antisense molecules could be expressed *in situ* from a single vector using two different promoters. For the reasons discussed in paragraphs 6 and 7, I would not have believed that the two antisense molecules described by Calabretta et al. would be produced with

similar efficiency. Indeed, no examples using this hypothetical construct are disclosed in the reference. Therefore, in April of 2000, a scientist working in this field would not have been motivated to combine with a reasonable expectation of success the disclosures of Calabretta *et al.* and Werther *et al.* with the disclosures of Fire *et al.* and Heifetz *et al.* to arrive at a vector expressing multiple double stranded RNAs.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

5-FEB-08
Date

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PROFESSIONAL EXPERIENCE:

NUCLEONICS INC, Horsham, PA 2002 - present

Director, Vector Technology and Preclinical Research

RNA interference (RNAi) vector development and analysis
Associate scientist supervision
Biosafety officer

Assistant Director

RNA interference (RNAi) vector development and analysis
Associate scientist supervision

Scientist

RNA interference (RNAi) vector development and analysis
In vivo study coordination

- Constructed plasmid DNA vectors designed to express double-stranded RNA
- Designed in vivo studies and maintained external collaborations designed to assess activity of RNAi vectors

GLAXOSMITHKLINE, Collegeville, PA 2001 – 2002

Principal Research Scientist

Transcriptome Analysis
Clotting Factor Analysis

- Used microarrays to determine host response to influenza virus infection
- GLP development and use of assays to measure clotting factor responses in sepsis

AXCELL BIOSCIENCES, Newtown, PA 2000 - 2001

Principal Research Scientist

Bacterial Protein Expression
cDNA Library Screening

- Developed methods for expression of recalcitrant protein domains.
- Used peptides to search for novel members of protein domain families.

WYETH-AYERST RESEARCH, Malvern, PA 1998 – 2000

Principal Research Scientist

Plasmid Vector Development
Associate Scientist Supervision
Vector Expression Analysis
Cytokine Expression Technology
Gene Expression Technology

- Developed expression system for analysis of bacterial ORF expression.
- Cloned genes for guinea pig IL-12.
- Developed plans for cytoplasmic expression system for DNA vaccines.

APOLLON, INC., Malvern, PA 1993 – 1998

Senior Research Scientist (1995-1998)

Plasmid Vector Development
Cytokine Vector Development
Vector Expression Analysis
External Collaboration Coordination
Biosafety Officer

- Constructed DNA vaccine vectors for viral and bacterial targets.
- Developed monocistronic and bicistronic plasmids designed to express cytokine genes.
- Developed tissue culture expression technologies to analyze protein expression from DNA vaccines.
- Developed external collaboration for the in vivo testing of candidate DNA vaccines.

Research Scientist (1993-1995)

Plasmid Vector Development
Vector Expression Analysis

- Constructed DNA vaccine vectors for retroviral targets including Rev-independent gag/pol and env expression.
- Developed competitive immunological assays to analyze tissue culture protein expression from DNA vaccines.

POST-DOCTORAL POSITIONS:

USDA-ARS-ERRC, Wyndmoor, PA 1991-1993 (Ching-Hsing Liao)
UNIVERSITY OF PENNSYLVANIA, Philadelphia 1989-1991 (David B. Weiner)

EDUCATION:

Ph.D., Immunology, CORNELL UNIVERSITY, Ithaca, NY 1989

M.S., Immunology, CORNELL UNIVERSITY, Ithaca, NY 1985

B.S., Microbiology, PENNSYLVANIA STATE UNIVERSITY, University Park, PA 1983

COMPUTER SKILLS:

Word Processing, Excel, PowerPoint, Internet

PROFESSIONAL AFFILIATIONS:

American Society for Microbiology

DANIEL E. McCALLUS
Addendum

ABSTRACTS

1. **McCallus DE and Norcross NL, 1986:** Experimental vaccine for coliform mastitis. Annual Meeting of the American Society for Microbiology.

McCallus DE and Norcross NL, 1987: Cross-rea

2. ctivity of *Escherichia coli* J5 antibodies with a smooth *E.coli* clinical isolate grown for different lengths of time. Annual meeting of the American Society for Microbiology.
3. **McCallus DE and Norcross NL, 1988:** Rabbit antiserum to *Escherichia coli* J5 cross-reacts with smooth *Escherichia coli* clinical isolates. Annual Meeting of the American Society for Microbiology.
4. **McCallus DE, Ugen KL, Williams WV and Weiner DB, 1991:** Expression of CD4 and HIV-specific immunoglobulins in bacteria. Annual Meeting of the American Society for Microbiology.
5. **McCallus DE and Liao C-H, 1992:** Biochemical and genetic characterization of protease from the soft-rotting bacterium *Pseudomonas fluorescens.* Sixth International Symposium on Molecular Plant-Microbe Interactions.
6. **Coney L, McCallus D, Wang B, Boyer J, Pachuk C, Higgins T, Carrano R, Sims D, Weiner D and Ciccarelli R, 1995:** Facilitated DNA injection elicits specific anti-HIV immune responses in preclinical animal models. Molecular Approaches to the Control of Infectious Diseases. Cold Spring Harbor, NY.
7. **McCallus DE, Higgins T, Wang B, Boyer J, Weiner D, Ciccarelli R and Coney L, 1996:** Injection of DNA containing SIV genes elicits specific immune responses in mice. 3rd Conference on Retroviruses and Opportunistic Infections. Washington DC.
8. **McCallus DE, Snyder LA, Herold KM, Pachuk CJ, Higgins T, Boyer J, Wang B, Weiner DB, Ciccarelli R and Coney LR, 1997:** Expression and immunogenicity of a DNA vaccine containing the genes for HIV-1 gag/pol. American Society for Microbiology, 97th General Meeting, Miami Beach, FL.

PUBLICATIONS

1. **McCallus DE and Norcross NL, 1987:** Antibody specific for *Escherichia coli* J5 cross-reacts to various degrees with an *Escherichia coli* clinical isolate grown for different lengths of time. Infect Immun. 55:1042-1046.
2. **Mohammed AH, McCallus DE and Norcross NL, 1988:** Development and evaluation of an enzyme-linked immunosorbent assay for endotoxin in milk. Vet Microbiol. 18:27-39.
3. **Weiner DB, McCallus DE, Williams WV and Greene MI, 1991:** Utilization of antiidiotypic antibodies as molecular probes of virus receptor interaction. Progress in Vaccinology Vol. 3: Anti-idiotype vaccines. P.-A. Cazenave, ed. Springer-Verlag, New York.
4. **McCallus DE, Ugen KE, Sato AI, Williams WV and Weiner DB, 1992:** Construction of a recombinant bacterial human CD4 expression system producing a bioactive CD4 molecule. Viral Immunology 5:163-172.
5. **Williams WV, Callegari P, Freundlich B, Keenan G, Eldridge D, Shin H, Dreitman M, McCallus D and Weiner DB, 1992:** Molecular diagnosis of *Borrelia burgdorferi* infection. DNA and Cell Biology 11:207-213.
6. **Ugen KE, McCallus DE, Von Feldt JM, Williams WV, Greene MI and Weiner DB, 1992:** Ocular tissue involvement in HIV infection: immunological and pathological aspects. Immunol Res. 11:141-153.
7. **Liao C-H, McCallus DE and Wells JM, 1993:** Molecular characterization of two gene loci required for production of the key pathogenicity factor pectate lyase in *Pseudomonas viridiflava.* Mol Plant Microbe Interact. 7:391-400.
8. **Kieber-Emmons T, von Feldt JM, Godillot AP, McCallus D, Srikantan V, Weiner DB, Williams WV, 1994:** Isolated VH4 heavy chain variable regions bind DNA characterization of a recombinant antibody heavy chain library derived from patient(s) with active SLE. Lupus. 3:379-392.

9. **Coney L, Wang B, Ugen KE, Boyer J, McCallus D, Srikantan V, Agadjanyan M, Pachuk CJ, Herold K, Merva M, Gilbert L, Deng K, Moelling K, Newman M, Williams WV, Weiner DB, 1994:** Facilitated DNA inoculation induces anti-HIV-1 immunity in vivo. Vaccine 12:1545-1550.
10. **Monfardini C, Kieber-Emmons T, Von Feldt JM, O'Malley B, Rosenbaum H, Godillot AP, Kaushshansky K, Brown CB, Voet D, McCallus DE, Weiner DB and Williams WV, 1995:** Recombinant antibodies in bioreactive peptide design. J. Biol Chem. 270:6628-6638.
11. **Wang B, Boyer J, Srikantan V, Ugen K, Agadjanyan M, Merva M, Gilbert L, Dang K, McCallus D, Moelling K, Carrano R, Williams WV, Coney L, Weiner DB,1995:** DNA inoculation induces cross clade anti-HIV-1 responses. Ann NY Acad Sci. 772:186-197.
12. **Liao C-H, McCallus DE, Wells JM, Tzean S-S and Kang G-Y, 1996:** The repB gene required for production of extracellular enzymes and fluorescent siderophores in *Pseudomonas viridflava* is an analog of the gacA gene of *Pseudomonas syringae*. Can. J. Microb. 42:177-182.
13. **Boyer JD, Ugen KE, Wang B, Agadjanyan M, Gilbert L, Bagarazzi ML, Chattergoon M, Frost P, Javadian A, Williams WV, Refaeli Y, Ciccarelli RB, McCallus D, Coney L and Weiner DB, 1997:** Protection of chimpanzees from high-dose heterologous HIV-1 challenge by DNA vaccination. Nat Med. 3:526-532.
14. **Liao C-H, McCallus DE, Fett WF, Kang Y 1997:** Identification of gene loci controlling pectate lyase production and soft-rot pathogenicity in *Pseudomonas marginalis*. Can J. Microb. 43:425-431.
15. **Liao, C-H, McCallus DE, 1998:** Biochemical and genetic characterization of an extracellular protease from *Pseudomonas fluorescens* CY091. Appl Enviorn Microbiol. 64:914-921.
16. **Kim JJ, Simbiri KA, Sin JI, Dang K, Oh J, Dentchev T, Lee D, Nottingham LK, Chalian AA, McCallus D, Ciccarelli R, Agadjanyan MG, Weiner DB, 1999:** Cytokine molecular adjuvants modulate immune responses induced by DNA vaccine constructs for HIV-1 and SIV. J. Interferon Cytokine Res. 19:77-84.
17. **Sin JI, Kim JJ, Arnold RL, Shroff KE, McCallus D, Pachuk C, McElhiney SP, Wolf MW, Pompa-de Bruin SJ, Higgins TJ, Ciccarelli RB, Weiner DB, 1999:** IL-12 gene as a DNA vaccine adjuvant in a herpes mouse model: IL-12 enhances Th1-type CD4+ T cell mediated protective immunity against herpes simplex virus-2 challenge. J Immunol. 162: 2912-2921.
18. **McCallus D, Pachuk C, Lee S, Satischandran C:** Current thoughts in DNA vaccines: delivery, safety and potential mechanisms of immune induction. In: New Vaccine Technologies. Editor: Ron Ellis, Landes Biosciences, Georgetown TX. In press.
19. **Pachuk C, McCallus D, Weiner DB, Satischandran C, 2000:** DNA vaccines – challenges in delivery. Current Opinion in Molecular Therapeutics.2:188-198.
20. **Sin JI, Kim J, Chattergoon M, Ayyavoo V, McCallus D, Ugen KE, Boyer JD, Weiner DB. 2000:** Engineering of DNA vaccines using molecular adjuvant plasmids. Dev Biol. 104:187-198.
21. **Shuey DJ, McCallus DE, Giordano T. 2002:** RNAi: gene-silencing in therapeutic intervention. Drug Discovery Today. 7:1040-1046.

ATTACHMENT B

MOLECULAR AND CELLULAR BIOLOGY, Nov. 1992, p. 5238–5248
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Interactions between Double-Stranded RNA Regulators and the Protein Kinase DAI

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The interferon-induced protein kinase DAI, the double-stranded RNA (dsRNA)-activated inhibitor of translation, plays a key role in regulating protein synthesis in higher cells. Once activated, in a process that involves autophosphorylation, it phosphorylates the initiation factor eIF-2, leading to inhibition of polypeptide chain initiation. The activity of DAI is controlled by RNA regulators, including dsRNA activators and highly structured single-stranded RNAs which block activation by dsRNA. To elucidate the mechanism of activation, we studied the interaction of DAI with RNA duplexes of discrete sizes. Molecules shorter than 30 bp fail to bind stably and do not activate the enzyme, but at high concentrations they prevent activation by long dsRNA. Molecules longer than 30 bp bind and activate the enzyme, with an efficiency that increases with increasing chain length, reaching a maximum at about 85 bp. These dsRNAs fail to activate at high concentrations and also prevent activation by long dsRNA. Analysis of complexes between dsRNA and DAI suggests that at maximal packing the enzyme interacts with as little as a single helical turn of dsRNA (11 bp) but under conditions that allow activation the binding site protects about 80 bp of duplex. When the RNA-binding site is fully occupied with an RNA activator, the complex appears to undergo a conformational change.

Protein synthesis is modulated at several levels, most commonly at the stage of polypeptide chain initiation, and the phosphorylation of initiation factors plays a key role in controlling this process (reviewed in references 19 and 20). In mammalian cells, a regulatory mechanism involving an RNA-activated protein kinase and the eukaryotic initiation factor 2 (eIF-2) has been intensively studied. This initiation factor forms a ternary complex with GTP and Met-tRNA$_F$ and delivers the initiator tRNA to the ribosomal site of protein synthesis initiation. Discharged eIF-2 is subsequently released as a complex with GDP which must be replaced with GTP to permit the formation of another ternary complex in preparation for a further round of initiation. The factor is composed of three dissimilar subunits, α, β, and γ. Phosphorylation of a single residue, serine-51 of the α subunit, inhibits translation by trapping a second initiation factor, the guanosine nucleotide exchange factor (or eIF-2B), which is required to catalyze the substitution of GTP for GDP in the discharged eIF-2 complex. Phosphorylation of sufficient eIF-2 can sequester all of the guanosine nucleotide exchange factor, thereby preventing eIF-2 recycling and halting the initiation pathway.

In mammals, two protein kinases are capable of phosphorylating the α subunit of eIF-2 in this way (reviewed in references 20, 37, and 46). One of them, the heme-controlled repressor, is found chiefly in reticulocytes. It is activated by the absence of hemin, as well as by other stimuli, and serves to prevent the accumulation of globin in the absence of iron or heme. A second kinase, the double-stranded RNA-activated inhibitor (DAI; also referred to as P1 kinase, p68 kinase, P1/eIF-2α kinase, and PK$_{ds}$, etc.) is present in a wide range of tissues. DAI is an important element in the host antiviral response, and its synthesis is induced at the transcriptional level by interferon (reviewed in references 21, 54, 56, and 59). The enzyme is ribosome associated (11, 34) and normally exists in an inactive or latent state. Under some circumstances, DAI activation leads to the virtually complete abrogation of protein synthesis, while in other circumstances it may contribute to the selective translation of particular classes of mRNA (8, 24, 26, 36, 47, 60). It has also been implicated in cellular differentiation (23, 52), in the inhibition of cell proliferation (6, 51), in the heat shock response (10), and possibly in transcriptional induction (61, 64). Moreover, in yeast cells, the related protein kinase GCN2 mediates the growth response to amino acid starvation (9). As its name implies, DAI is activated by double-stranded RNA (dsRNA). Other polyanions such as heparin can also activate it, while small, highly structured RNA molecules such as adenovirus VA RNA suppress its activation (38). Thus, DAI is a pivotal cellular regulatory enzyme whose level and activity are modulated by factors of both viral and cellular origin.

The interactions between DAI and its RNA effectors are complicated and incompletely understood. The kinase is activated by dsRNA but not by DNA or DNA-RNA hybrids (22, 32, 35, 58). Single-stranded RNA, either synthetic or natural, is also inactive unless it can form extended hairpin-like structures (5, 22). There is no discernible sequence dependence for activation by dsRNA, and limited mismatching (44) and some modified bases (2, 45, 62) are tolerated, but the activity of dsRNA is reduced by ethidium bromide (1), suggesting that the topological form of the RNA duplex is important. Activation is accompanied by autophosphorylation of the kinase at multiple sites on serine and threonine residues (3, 11, 14, 30), and results in a change of substrate specificity such that the activated enzyme can phosphorylate the α subunit of eIF-2 and some other proteins (53, 58) but can no longer phosphorylate other molecules of DAI (29). Once activated, however, the phosphorylated enzyme is unaffected by the addition or removal of dsRNA (11, 58, 63).

Activation of DAI by dsRNA displays a paradoxical concentration dependence: the enzyme is activated by low concentrations of dsRNA (in the range of 10 to 100 ng/ml), but higher concentrations are decreasingly effective activators, giving rise to a bell-shaped activation curve (11, 22, 27,

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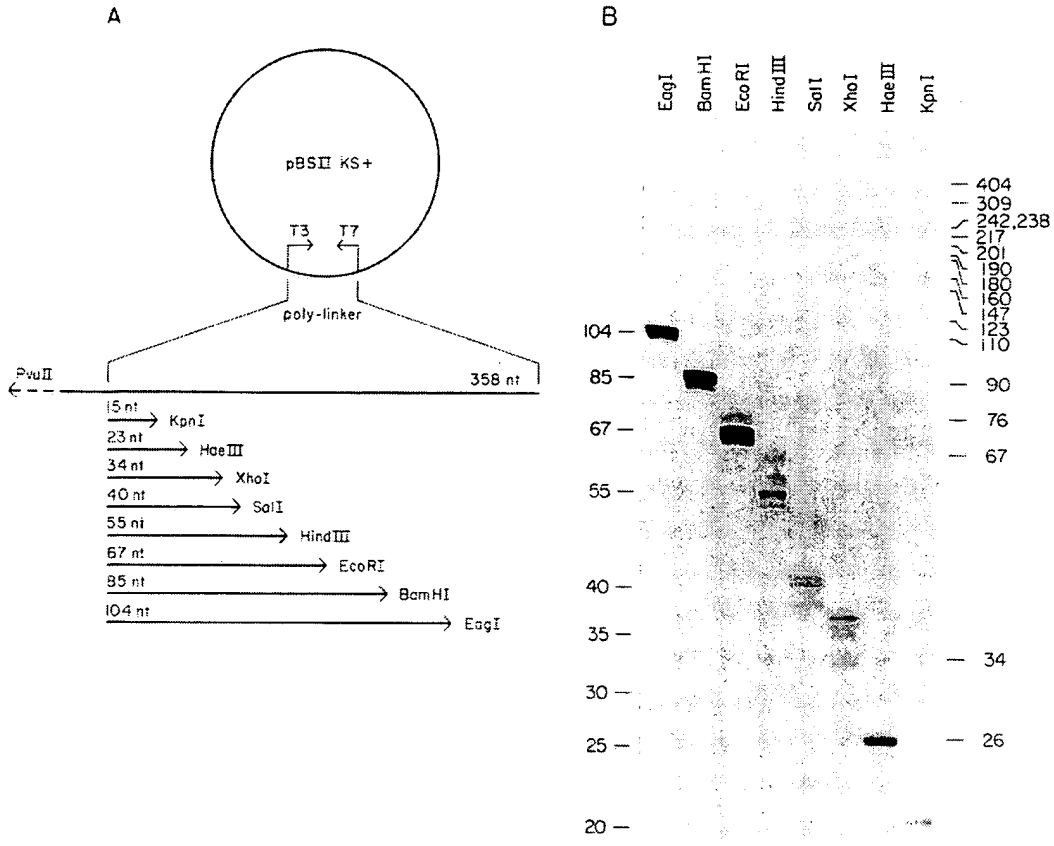

FIG. 1. Synthesis and characterization of RNA duplexes. (A) Schematic of short dsRNAs produced by transcription of pBSII KS+ polylinker sequences. The several transcripts of 15 to 104 nt synthesized by T3 RNA polymerase (rightward arrows) were annealed to the complementary 358-nt transcript (leftward arrows) synthesized by T7 RNA polymerase. After RNase digestion, the duplexes were purified by electrophoresis in nondenaturing gels. (B) Analysis of the purified dsRNAs in denaturing conditions. Samples of the radiolabeled dsRNAs were heated in formamide and resolved by electrophoresis in a 10% polyacrylamide–7 M urea gel. The fixed and dried gel was subjected to autoradiography. Size markers were single-stranded RNAs synthesized as described in part A (left) and pBR322-*Hpa*II DNA fragments (right).

33). High concentrations of dsRNA prevent the activation process but do not interfere with the activity of DAI once it has been activated by dsRNA at a lower concentration. The kinase also displays a stringent requirement for dsRNA chain length. Activation is reported to require a minimum of about 50 bp of duplex (18, 22, 32, 44), and there are indications that shorter duplexes may block activation at high concentration (44), as long dsRNA does. The ability of the enzyme to discriminate between dsRNA molecules on the basis of their chain length has implications for its regulation and the mechanism of DAI activation (37). Here we investigate the interactions of the enzyme with dsRNA molecules of specified sizes, studying binding and protection of dsRNA as well as activation and inhibition of the kinase. Our results define the dsRNA size dependence of the interaction and confirm that short duplexes which fail to bind stably and to activate the kinase can still interfere with activation mediated by longer duplexes. The data suggest that the minimal recognition element is a single helical turn but that there is an extended site for dsRNA binding which needs to be completely occupied for full enzyme activation.

MATERIALS AND METHODS

Synthesis of short dsRNAs. The plasmid pBSII KS+ (Stratagene, Inc., La Jolla, Calif.) was banded twice in CsCl, passed over a Bio-Gel A 15-m column, and then digested with one of eight enzymes (*Kpn*I, *Hae*III, *Xho*I, *Sal*I, *Hin*dIII, *Eco*RI, *Bam*HI, or *Eag*I), which cut in the polylinker, or with *Pvu*II, which cuts outside the region containing the polylinker and the T3 and T7 promoters (Fig. 1A). The DNA was incubated with RNase A to remove the last traces of RNA, treated with proteinase K, and extracted with phenol and chloroform. After ethanol precipitation, the DNA was added to transcription reactions containing T7 RNA polymerase (17) for the PvuII-digested template or T3 RNA polymerase (Stratagene, Inc.) for the other templates. Reaction conditions were as described previously (43), except that the concentration of GTP or CTP was reduced to 12 μM for labeling. The corresponding [α-^{32}P]ribonucleotide (from ICN Biomedicals Inc., Costa Mesa, Calif.) was present at a concentration of 500 μCi/ml. Single-stranded RNA was recovered after DNaseI digestion and phenol and chloroform extraction by ethanol precipitation. Each of the

T3 products (15 to 104 nucleotides [nt]) was mixed with an approximately equivalent amount of the complementary T7 product (354 nt), heated to 100°C, and annealed as previously described (29). Following digestion with both RNase T_1 and RNase A, dsRNA was isolated by treatment with proteinase K and deproteinization and then fractionated by electrophoresis in a nondenaturing 10% polyacrylamide–0.5× Tris-borate-EDTA (TBE) gel. The bands were detected autoradiographically, and each dsRNA was eluted into 10 mM Tris–1 mM EDTA –10 mM NaCl–0.5% sodium dodecyl sulfate (SDS), deproteinized, and ethanol precipitated. The dsRNA was dissolved in the same buffer without SDS, and its concentration was calculated from the specific activity.

Other RNAs. Longer dsRNAs (354 bp) were synthesized by transcription of the pGEM.GC plasmid (42). Reovirus dsRNA was provided by A. J. Shatkin (Rutgers University, New Brunswick, N.J.), and *Penicillium chrysogenum* and bacteriophage f2 sus3 dsRNAs were provided by H. D. Robertson (Cornell University Medical School, Ithaca, N.Y.). Labeled single-stranded RNA was purified from the T7 and T3 polymerase transcription reactions described above by electrophoresis through a 10% polyacrylamide–7 M urea–0.5× TBE gel.

Kinase assays. Reactions (10 μl) containing 2.5 μCi of [γ-^{32}P]ATP (ICN Biomedicals, Inc.) and 0.5 μl of DAI (about 5 ng) purified to the Mono S stage (29) were conducted essentially as described by Mellits et al. (42). The enzyme was added last to the other reaction components assembled on ice. Phosphorylation was visualized by SDS-polyacrylamide gel electrophoresis and autoradiography for 4 to 16 h by using an intensifier screen.

Nitrocellulose filter-binding assay. The nitrocellulose filter-binding assay was conducted by using a modification of the published procedure of Kostura and Mathews (29). Briefly, labeled dsRNA was incubated for 20 min on ice with the Mono S fraction of DAI under kinase reaction conditions, with bovine serum albumin (BSA) and calf liver tRNA both added to a concentration of 0.1 mg/ml but with labeled ATP omitted. After dilution with 10 volumes of wash buffer (50 mM KCl, 1.5 mM $MgCl_2$, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) K^+ [pH 7.4], 0.1 mM EDTA), the reaction mixtures were immediately filtered in a slot-blot apparatus through a 0.45-μm-pore-size nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) that had been soaked for 1 h at room temperature in wash buffer containing 0.1 mg each of BSA and salmon sperm DNA per ml. Each well was washed with 200 μl of ice-cold wash buffer, and the filter was dried and exposed to autoradiography. Quantitation was done by scintillation counting of individual bands or direct scanning of the membrane with the AMBIS Imaging System.

Binding of dsRNA to Sepharose-bound DAI. A mixture of dsRNAs was partially degraded by incubation with RNase T_1 and RNaseIII (provided by H. D. Robertson) and then incubated with DAI immobilized on monoclonal antibody-Sepharose beads (13, 31) (from A. Hovanessian, Institut Pasteur, Paris, France) as described previously (40). The beads were sedimented and washed five times by resuspension and sedimentation. Of the input radioactivity, approximately 15% was recovered with the beads, 80% was recovered in the initial supernatant plus first wash fraction and 5% was recovered in subsequent washes. RNA was extracted from the beads and from the initial supernatant plus the first wash fraction and was analyzed by electrophoresis in a nondenaturing 10% polyacrylamide–0.5× TBE gel.

Protection of dsRNA by DAI. Radiolabeled dsRNA (354 bp) was bound to immobilized DAI as described above. After the third wash, the beads were washed twice with RNaseIII buffer (100 mM NH_4Cl, 10 mM magnesium acetate, 20 mM Tris-HCl [pH 7.6]) and then incubated with 50 U of RNaseIII per ml for 30 min at 37°C. An equal amount of fresh RNaseIII was added, and the incubation continued for a further 30 min. RNA was isolated from the beads and from the supernatant fractions and analyzed as described above.

Gel retardation assay. Binding reactions (10 μl) were similar to those for kinase assays, except that ATP was omitted and tRNA and BSA were present at 0.1 and 1 mg/ml, respectively. The concentration of labeled dsRNA was 55 ng/ml, and the concentration of DAI (Mono S fraction), immunoaffinity chromatography-purified DAI (14), or p20 (55) was varied. After incubation for 20 min on ice, a dye-glycerol solution was added and the samples were loaded directly onto a 5% polyacrylamide gel (acrylamide: bioacrylamide, 82:1). The gel was cast in 40 mM Tris-glycine buffer and had been prerun for 1 h at 150 V. Radioactivity was detected by autoradiography for approximately 16 h.

RESULTS

Characteristics of synthetic dsRNA. Duplexed RNAs of defined sizes were made by annealing a 358-nt transcript synthesized by T7 RNA polymerase with complementary transcripts of various lengths synthesized by T3 RNA polymerase (Fig. 1A). After digestion of the RNA tails and residual single-stranded RNA, the dsRNAs were purified by electrophoresis in nondenaturing polyacrylamide gels. When analyzed in denaturing conditions (Fig. 1B), the individual strands of the dsRNA molecules were slightly heterogeneous, with chain lengths a few nucleotides longer or shorter than the input single strands as a result of the trimming process. When examined in a nondenaturing gel, however, the dsRNAs migrated as discrete bands, with mobilities similar to those of dsDNA markers (see Fig. 5A, lanes 3 to 9). As expected, the duplexes were sensitive to digestion with RNaseIII, a dsRNA-specific enzyme, but resistant to digestion by single-stranded specific nucleases except after denaturation (data not shown).

Activation and inhibition of DAI. Activation of DAI is accompanied by its autophosphorylation, converting the enzyme from a latent state to a form which can phosphorylate eIF-2α. When the synthetic duplexes were examined for their ability to catalyze autophosphorylation, we found that 23- and 34-bp dsRNAs were only slightly active, 40-bp dsRNA was partly active, and full activity was approached with 55- to 85-bp dsRNAs, which were nearly as active as the very long dsRNA (average size of >2,000 bp) isolated from reovirus virions (Fig. 2A). These results, obtained with essentially flush-ended dsRNA, agree closely with previously reported data obtained by using RNA molecules in which one strand was considerably longer than the other (44): in the earlier study, duplexes shorter than 30 bp were unable to activate DAI, and full activation was obtained with duplexes longer than 65 to 80 bp. The activation of DAI can also be monitored by phosphorylation of eIF-2, the natural substrate of this kinase. In this assay, 15-bp dsRNA was essentially inactive, 34-bp dsRNA was partially active, and 55-bp (or longer) dsRNA was fully active (Fig. 2B). Thus, the slight autophosphorylation of DAI that is catalyzed by the 34-bp duplex is sufficient to permit DAI to phosphorylate its natural substrate weakly. These results are consistent with the findings that the very short (<20 bp) imperfect duplexes found in viral RNAs such as VA RNA (28, 38, 41,

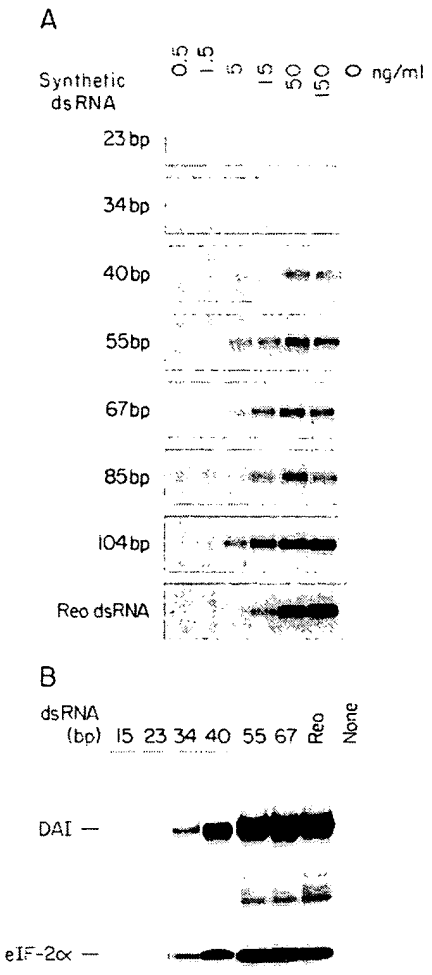

FIG. 2. Activation of DAI by short duplexes. (A) Autophosphorylation of DAI as a function of dsRNA concentration and chain length. Kinase assay reaction mixtures containing synthetic or reovirus dsRNA at the concentrations indicated were analyzed by electrophoresis in SDS-polyacrylamide gels and autoradiography. (B) Phosphorylation of eIF-2. Kinase assay reaction mixtures containing 33 ng of the synthetic dsRNAs indicated per ml or 40 ng of reovirus dsRNA per ml were supplemented with eIF-2 and were analyzed as described in part A. The positions of autophosphorylated DAI and the phosphorylated α subunit of eIF-2 are marked.

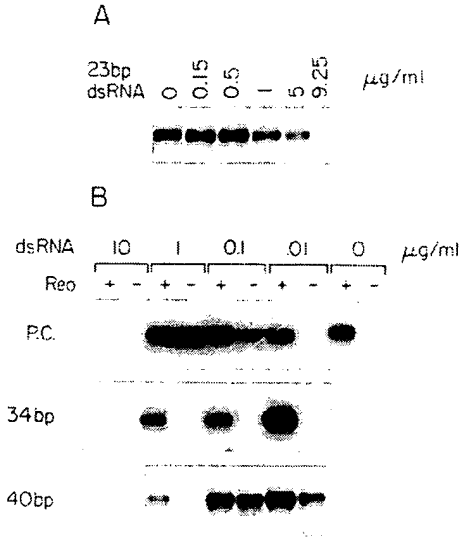

FIG. 3. Inhibition of DAI activation by high concentrations of dsRNA. (A) Inhibition of DAI autophosphorylation by 23-bp dsRNA. Kinase assay reaction mixtures contained 20 ng of reovirus dsRNA per ml and the indicated concentrations of the 23-bp dsRNA. (B) Effect of duplexes of various lengths on DAI autophosphorylation in the presence and absence of an activator dsRNA. Kinase assay reaction mixtures containing or lacking reovirus dsRNA (40 ng/ml) were supplemented with synthetic 34- or 40-bp dsRNAs or with *P. chrysogenum* dsRNA (P.C.) at the concentrations indicated. Autophosphorylation of DAI was assessed as described for Fig. 2.

42, 48), EBER (4, 7) and TAR RNA (18) are insufficient to cause DAI activation.

From experiments with tailed duplexes, it was concluded that short dsRNAs which are incapable of activating DAI may nevertheless block the activation of DAI by longer dsRNAs (44). Figure 3A shows that at high concentration a flush-ended 23-bp dsRNA inhibited the activation of DAI by reovirus dsRNA. In this respect, short dsRNA resembles longer dsRNA molecules which block DAI activation at high concentrations, although the mechanisms might be different. Figure 3B demonstrates that long dsRNA (approximately 3,000 bp) isolated from *P. chrysogenum* activates DAI at concentrations of up to 1 μg/ml and prevents activation at 10 μg/ml, as expected from the bell-shaped activation curve. Short duplexes, such as the 34-bp dsRNA (Fig. 3B), activate DAI only weakly and also become inhibitory at approximately 10 μg/ml. Similar results were obtained with bacteriophage f2 sus3 dsRNA (approximately 30 to 50 bp long [22]) and the 23-bp synthetic duplexes (data not shown), while the 40-bp dsRNA gave a response intermediate between that of the longer and shorter duplexes because of its significant ability to activate DAI (Fig. 3B). Single-stranded RNA is not inhibitory (data not shown). These results confirm that the enzyme is activated by relatively long dsRNA and can interact with short duplexes in a nonproductive way.

RNA size dependence of DAI binding. The existence of opposing effects of dsRNA on DAI activity makes it important to address directly the binding of this ligand to the protein. We examined the interactions between DAI and dsRNA, using three assays which explore different aspects of the process: nitrocellulose filter binding, binding to an affinity matrix, and gel retardation. Figure 4A and B illustrates the binding of single-stranded RNA and dsRNA as a function of chain length in the filtration assay. Duplexes with sizes of 15 and 23 bp did not bind detectably, whereas 34- and 40-bp duplexes bound weakly. Longer molecules bound with increasing efficiency, and 85- or 104-bp dsRNA bound as efficiently as 354-bp molecules. Single-stranded molecules bound only very weakly, except for the 354-nt molecule, which may be able to form a small amount of secondary structure. The binding of dsRNAs to DAI was also measured as a function of ligand concentration (Fig. 4C and D). The efficiency with which short duplexes bound to DAI did not improve at subsaturating RNA concentrations, suggesting that the affinity of the enzyme is low for molecules with sizes

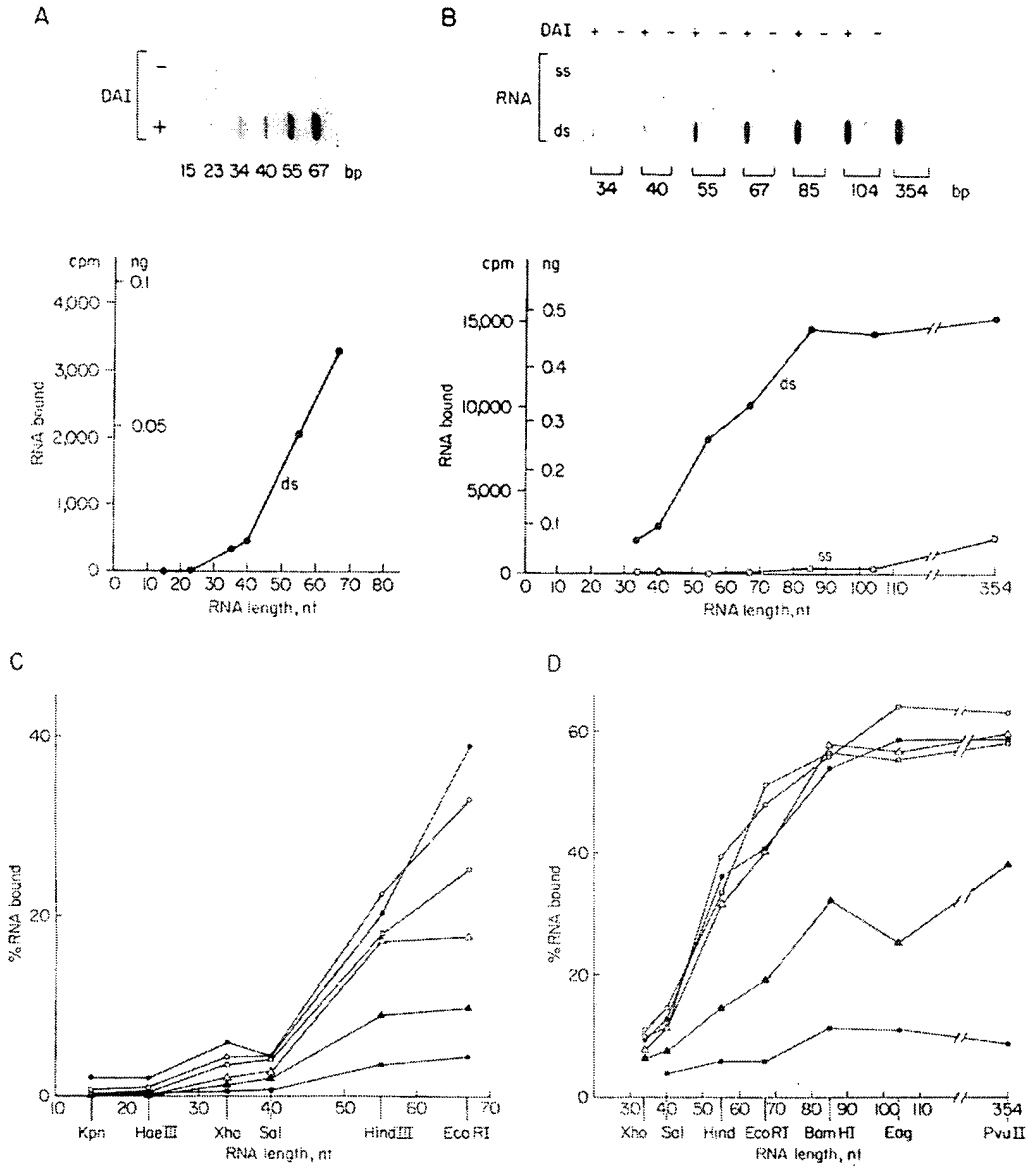

FIG. 4. Size dependence of dsRNA binding to DAI. (A and B) Nitrocellulose filter-binding assay for the binding of single-stranded RNA and dsRNAs. Autoradiograms of the membrane are shown (top panel). Quantitation of RNA binding (bottom panel) was obtained by scintillation counting or scanning of the individual filter bands and subtraction of the background value (lanes labeled −DAI) from the signal radioactivity (lanes labeled +DAI). ●, dsRNA (ds); ○, single-stranded RNA (ss). (C and D) Concentration and size dependence of dsRNA-binding efficiency. The percentage of the input dsRNA that was retained on the filter in the presence of DAI was quantified at various dsRNA concentrations of 1,000 (■), 330 (▲), 100 (△), 33 (□), 10 (○), and 3.3 (●) ng/ml, respectively.

of 40 bp or less and that the affinity increases uniformly as the chain length is increased, reaching a maximum at 85 bp. These data agree closely with the dependence of activation and inhibition on dsRNA chain length (Figs. 2 and 3) and are consistent with a model which equates activation with stable dsRNA binding. Duplexes shorter than 30 to 40 bp bind weakly and cannot activate although they inhibit activation; longer duplexes (40 to 85 bp) bind with increasing stability and their ability to activate the enzyme increases concomitantly; beyond this length, the efficiency of binding and activation remains unchanged.

Binding and protection of dsRNA. One interpretation of these observations is that the dsRNA binding site in DAI accommodates up to ~85 bp of duplex but can bind shorter duplexes less stably, down to ~30 bp. To test this interpretation we employed DAI immobilized on antibody-Sepharose beads. First, to define the minimum size of dsRNA that can bind to the enzyme, a mixture of dsRNA molecules was partially digested with RNaseIII to generate a collection of duplex molecules with a broad size distribution. This collection was allowed to bind to the immobilized DAI, and the beads were washed to remove nonspecifically adsorbed dsRNA. Figure 5A displays the DAI-bound dsRNA (lane 1) and the unbound RNA that remained in the supernatant (lane

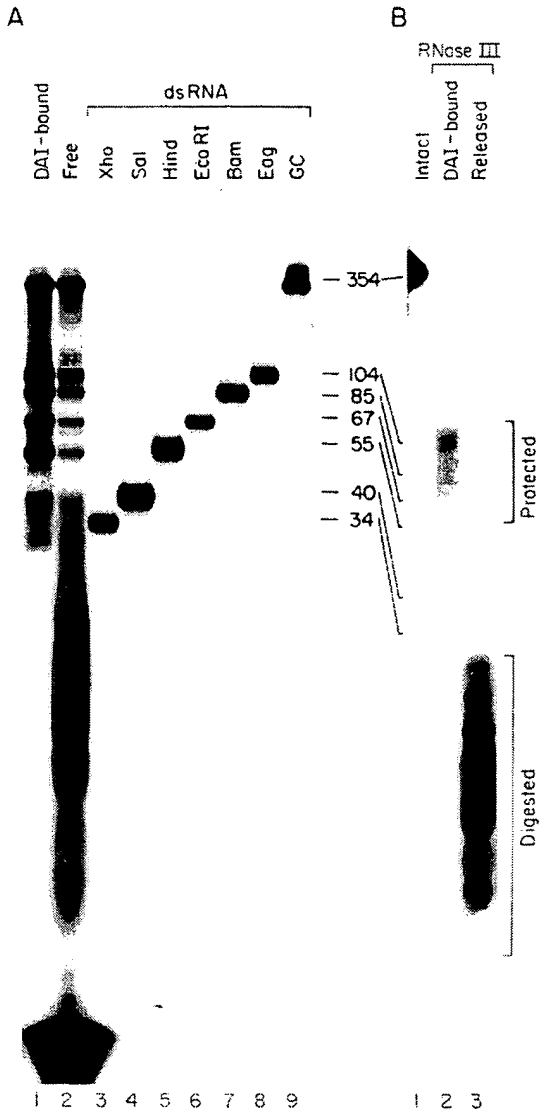

FIG. 5. Binding and protection of dsRNA fragments by DAI. (A) Binding of randomly sized dsRNA fragments. A mixture of fragments (approximately 10 to 350 bp) was incubated with DAI attached to Sepharose beads. Unbound RNA was separated from the beads by centrifugation and washing. Equal fractions of the DAI-bound (lane 1) and unbound (lane 2) dsRNA were resolved in a nondenaturing polyacrylamide gel and detected by autoradiography. Markers included discrete dsRNA fragments with sizes of 34 to 104 bp (lanes 3 to 8), denoted by the restriction site designation used in their synthesis (Fig. 1A), and 354 bp (lane 9), denoted GC for the plasmid used in its synthesis (pGEM.GC). (B) Protection of DAI-bound dsRNA from digestion by RNaseIII. Discrete 354-bp dsRNA (lane 1) was bound to DAI attached to Sepharose beads, and unbound RNA was removed. The beads were exhaustively incubated with RNaseIII, and the released RNA fragments were collected. Equal fractions of the released RNA (lane 3) and the RNA that remained associated with the beads (lane 2) were resolved as described for panel A.

2). Comparison with dsRNA markers (lanes 3 to 9) and with an RNA sequence ladder (data not shown), indicated that the cutoff for binding was at approximately 28 bp, in good agreement with results obtained in the nitrocellulose filter-binding assay. Moreover, visual inspection of the autoradiogram suggested that dsRNA with a size of 28 to 40 bp bound less efficiently than longer duplexes.

Next, we conducted a protection experiment to determine the length of dsRNA that is shielded by DAI from nuclease attack. Intact 354-bp dsRNA was bound to DAI immobilized on antibody-Sepharose beads, and the excess unbound dsRNA was removed. The bound dsRNA was digested by incubating the beads with RNaseIII to trim off regions of duplex that were not protected by DAI. Figure 5B, lane 3, shows that the released dsRNA had been reduced to fragments of approximately 10 to 20 bp as expected (57), whereas the bulk of the DAI-associated material (lane 2) ranged in size from approximately 60 to 120 bp, with a substantial concentration in the longer-size class (approximately 100 to 120 bp). Assuming that RNaseIII leaves 15 bp of dsRNA protruding on each side, we deduce that DAI associates with 30 to 90 bp of dsRNA. Taking 110 bp as the modal length of the protected fragments, it appears that about 80 bp of duplex interact directly with the enzyme, roughly the length of dsRNA that gives maximal binding in the nitrocellulose filter assay. The length of the protected fragment was not altered at relatively high concentrations of dsRNA (up to 1 μg/ml; data not shown), conditions which would be expected to disfavor oligomerization of DAI on the dsRNA. These findings support the view that the dsRNA site extends for ~80 bp and that shorter molecules bind with lesser affinity, provided that they are at least 28 bp long.

DAI-dsRNA complexes. To characterize the interactions more directly, we examined complexes formed between DAI and dsRNAs in a gel retardation assay (Fig. 6A). No complexes were observed with 15- or 23-bp duplexes (data not shown), but longer dsRNAs formed complexes with increasing efficiency. Four series of complexes were distinguishable (bands I to IV). Their relative abundance was principally a function of RNA chain length, with a lesser dependence on DAI concentration. On the basis of their behavior, the complexes seem to fall into two families. One family, containing the more slowly moving bands I and II, forms preferentially with duplexes of less than optimal length (34 to 67 bp) in binding and activation assays. The second family, containing the faster moving bands III and IV, forms preferentially with longer duplexes (≥85 bp), which are fully active in binding and activating DAI.

Band I was the most prominent complex with 55- and 67-bp duplexes but was barely detectable with longer or shorter duplexes. It was formed at low DAI concentrations and seemed to be converted to band II at elevated DAI concentrations. Indeed, regardless of chain length, complex II was seen only at high DAI concentrations. With 34- and 40-bp duplexes, the only detectable complexes appeared to migrate in band II. Formation of complex II increased as the chain length was extended, reaching maximal levels with the 67-bp duplex and declining as the chain length was extended further, to 85 and 104 bp.

The most abundant complexes, formed with 85- and 104-bp duplexes, migrated in band III. This band was also visible with 67-bp and perhaps 55-bp dsRNAs. As chain length increased, complex III was formed at progressively lower concentrations of DAI. It also seemed to decrease slightly at high DAI concentrations. Band IV displayed a pattern similar to that of band III but was always less

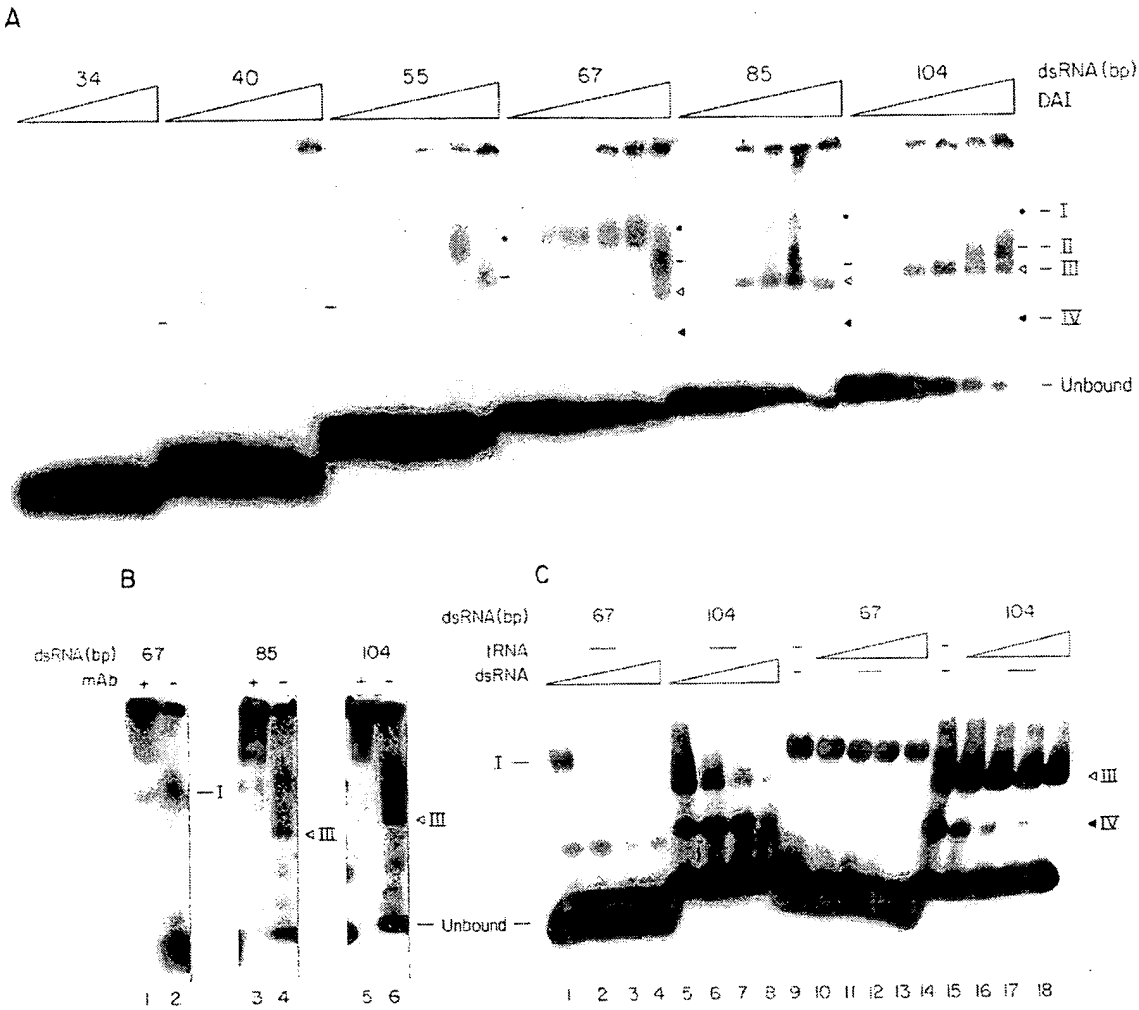

FIG. 6. Gel-shift analysis with DAI. (A) Dependence on dsRNA size and DAI concentration. dsRNAs with sizes of 34 to 104 bp were incubated with various amounts of DAI (0, 0.05, 0.1, 0.25, 0.5, and 1.0 μl from left to right, symbolized by the wedges) purified to the Mono S stage. The resultant complexes were separated by electrophoresis in nondenaturing conditions and detected by autoradiography. The positions of complexes I (●), II (—), III (◁), and IV (◀) and of the free dsRNA fragments are marked in each panel. (B) Shifts with essentially homogeneous DAI and antibody supershift. dsRNA with a size of 67 bp (lanes 1 and 2), 85 bp (lanes 3 and 4), or 104 bp (lanes 5 and 6) was incubated with 1 μl of DAI purified by immunoaffinity chromatography in the presence (lanes 1, 3, and 5) or absence (lanes 2, 4, and 6) of 1 μl of monoclonal antibody to DAI. (C) Competition assays. Standard reaction mixtures (lanes 9 and 14) contained DAI (Mono S fraction) and 100 μg of tRNA per ml; *P. chrysogenum* dsRNA (0.25, 0.5, 0.75, and 1 μg/ml; lanes 1 to 4 and 5 to 8) or additional calf liver tRNA (100, 200, 300 and 400 μg/ml; lanes 10 to 13 and 15 to 18) was added as indicated. ^{32}P-labeled dsRNA (67 bp [lanes 1 to 4 and 9 to 13] or 104 bp [lanes 5 to 8 and 14 to 18]) was present at 55 ng/ml. The wedges symbolize increasing concentrations from left to right; — indicates absence of the RNA.

abundant. Both of these bands correlate well with full enzyme activity.

Very similar patterns of bands were formed with an essentially homogeneous preparation of DAI purified by immunoaffinity chromatography (Fig. 6B), and the gel-shift activity cosedimented with kinase activity through a glycerol gradient (29) (data not shown). Furthermore, as seen in Fig. 6B, all of the complexes were "supershifted" to forms with slower mobility by addition of monoclonal antibody directed against DAI. The antibody did not produce a gel shift on its own (i.e., in the absence of DAI), but it appeared to stabilize DAI-dsRNA complexes so that less probe remained in free form. These experiments verified that bands I to IV all contain DAI. Competition experiments demonstrated that the most prominent complexes, band I with 67-bp dsRNA and band III with 104-bp dsRNA, were resistant to the presence of excess tRNA but were sensitive to unlabeled *P. chrysogenum* dsRNA competitor (Fig. 6C). The complexes formed with the 104-bp dsRNA were more resistant to competition than those formed with 67-bp dsRNA, as expected from the higher affinity of DAI for longer duplexes (Fig. 4). For some of the minor bands (e.g., band IV), competition was more effective with tRNA at very high concentrations than with dsRNA at moderate concentrations

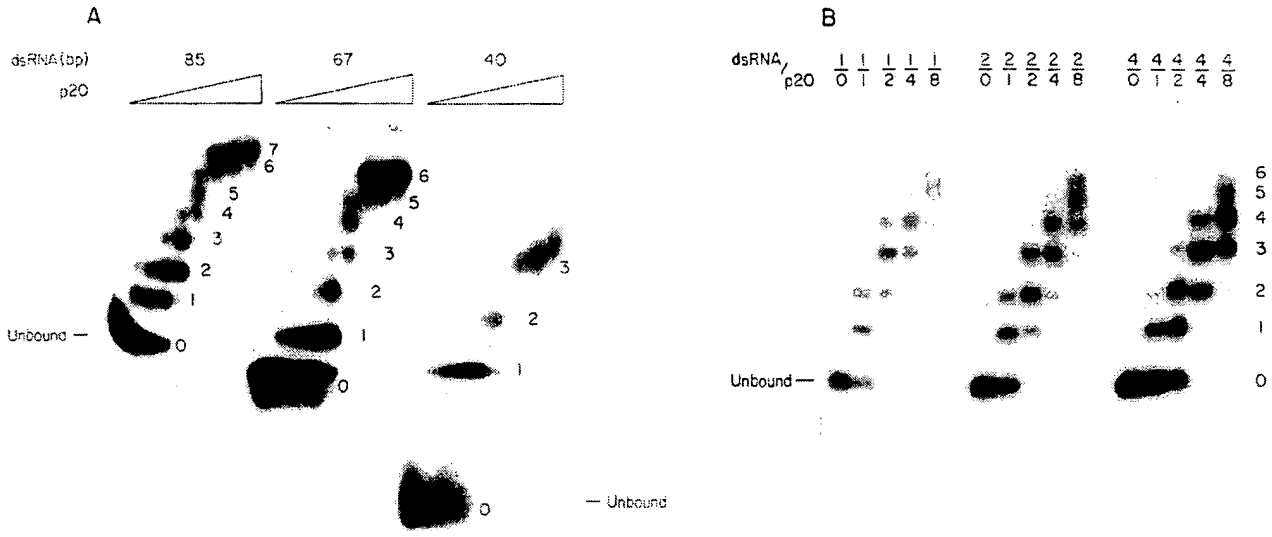

FIG. 7. Gel shift analysis with the p20 polypeptide. (A) Dependence on dsRNA size. Reaction mixtures contained dsRNA of 40, 67, or 85 bp as indicated and 0, 0.032, 0.063, 0.125, 0.25, 0.5, 2.5, 5, or 10 μg/ml of p20 (increasing from left to right as symbolized by the wedges). The number of p20 units in each band is indicated on the right. (B) Dependence on dsRNA and p20 concentration. Assay mixtures contained 85-bp dsRNA at a concentration of 55, 110, or 220 μg/ml and p20 at a concentration of 0, 63, 125, 250, or 500 ng/ml. Concentrations increase from left to right. The relative concentrations of these two components are indicated above the autoradiogram, and the number of p20 units in each band is marked on the side.

for reasons that are presently unclear. Neither single-stranded DNA nor dsDNA was an effective competitor (data not shown). These results demonstrate that the most prominent complexes are dsRNA and DAI specific and that there is a rather abrupt change in electrophoretic mobility when the dsRNA reaches the size for optimal binding and activation (approximately 80 bp).

Minimal binding site. The ability of short duplexes (<30 bp) to inhibit DAI activation implies that they interact with the enzyme, even though their binding is not sufficiently stable to be detected by the assays used to this point. To determine the minimal length of duplex that can interact with DAI, we employed the close-packing method for estimating the number of protein molecules that can bind to duplexes with known sizes. For this purpose, we used a truncated version of DAI, p20, comprising the N-terminal 184 amino acids which we and others have determined to contain the RNA-binding domain of the protein (16, 25, 39, 50). The numbers of p20 molecules binding to a given dsRNA were estimated from gel-shift assays conducted at increasing concentrations of the protein. As seen in Fig. 7A, a series of complexes was formed, reaching a maximum at the highest protein levels. The number of complexes increased with increasing dsRNA chain length as follows: 40 bp, three bands; 67 bp, six bands; 85 bp, seven bands. Assuming that each shifted band corresponds to the binding of a p20 molecule, these data imply that the minimum binding site is about 11 bp, equivalent to a single turn of A-form RNA helix.

The appearance of a ladder of bands with p20 suggested that there may be differences between the binding of this fragment and intact DAI to dsRNA. Further experiments showed that the formation of the p20 complexes is specific in that tRNA does not compete (data not shown). To rule out the possibility that the p20 banding patterns are due to a concentration-dependent protein oligomerization that is independent of dsRNA, we conducted band-shift assays at increasing dsRNA concentrations. Figure 7B shows that more p20 protein is required to achieve a given gel shift at higher concentrations of dsRNA (compare, for example, the amount of p20 needed to complex all of the dsRNA in the reactions). This indicates that oligomerization depends on the presence of dsRNA and is due to the formation of a series of protein-RNA complexes rather than to preformed protein-protein aggregates. The banding pattern was also influenced by the absolute concentration of p20 and dsRNA, however, as can be seen by comparing lanes with equal ratios of dsRNA to p20 (such as 1/1, 2/2, and 4/4). The shift to larger complexes at higher concentrations could merely reflect the concentration dependence of the reaction according to the law of mass action, or it could imply that p20 complexes are stabilized by protein-protein interactions when p20 monomers are bound adjacently on dsRNA. In the latter case, stabilizing protein-protein interactions would provide an explanation for the apparent paradox that DAI binds efficiently only to duplexes of longer than 30 bp but can bind to as little as a single helical turn of dsRNA.

DISCUSSION

Although the existence of DAI has been known for many years and its activation by a variety of polynucleotides has been studied intensively, an understanding of the enzyme's regulation has remained elusive. The kinase is activated by autophosphorylation in the presence of dsRNA. This response exhibits a number of unusual features: first, activation is prevented by high concentrations of dsRNAs which activate the kinase at low concentrations; second, short RNA duplexes fail to activate DAI at any concentration but prevent activation at elevated concentrations; third, highly structured single-stranded RNAs of viral origin also fail to activate DAI but can block activation by authentic, long

dsRNA. To illuminate the interactions between dsRNA and DAI, we generated a series of short RNA duplexes and studied directly their binding to the enzyme as well as their effects on its activity. The results correlate activation with the formation of stable complexes with a characteristic electrophoretic mobility and suggest a model that is compatible with the emerging understanding of DAI structure.

Our results are most consistent with the view that DAI possesses a single effective site for dsRNA, capable of accommodating approximately 80 bp of duplex. Two observations support this conclusion most strongly. First, as the length of the dsRNA ligand is increased, maximal binding is attained at this size and longer molecules bind no more efficiently, and second, the kinase protects this length of duplex from digestion by nuclease. Shorter duplexes, down to a lower limit of approximately 30 bp, bind with steadily decreasing efficiency while duplexes with lengths of less than 30 bp are unable to form a stable complex with DAI under normal conditions. Nonetheless, since such very short duplexes block the activation of DAI, we assume that at high concentrations they form transient interactions which prevent DAI activation. Likewise, other polynucleotides, such as RNA-DNA hybrids and partially methylated dsRNA duplexes that fail to activate the kinase, as well as long dsRNAs that can activate DAI, share this property of inhibiting kinase activation at high concentrations. The nature of these inhibitory interactions is unclear, and it remains to be seen whether viral effectors such as VA RNA, EBER, and TAR RNA function in the same way as short duplexes or whether they interact in a distinct fashion to block DAI activation. Preliminary data indicate that the sites for VA RNA and dsRNA are overlapping but perhaps not congruent (16).

How do these functional observations relate to the structure of the enzyme? DAI possesses two RNA-binding elements in its N-terminal domain (12, 16, 25, 39, 50). Each element contains an RNA-binding motif which is rich in basic amino acids and is predicted to form an α-helical structure (16, 39). Both elements are required for efficient binding of RNA, and they appear to cooperate to form a single bivalent site which optimally extends over approximately 80 bp of duplex. Since the RNA binding domain of DAI, expressed as the p20 protein, is able to interact with as little as 11 bp of dsRNA, we speculate that each element interacts with a single helical turn and that optimal binding occurs when these two turns are separated by about five intervening helical turns. In this complex, the entire span of approximately 80 bp is protected by DAI against attack by the dsRNA-specific nuclease RNaseIII. With this model, interactions with shorter dsRNA molecules entail increasing strain on the enzyme, accompanied by decreasing affinity, such that it becomes impossible for both elements to bind when there is less than one intervening helical turn (at approximately 33 bp). Evidently, monovalent complexes can also be formed at high ratios of enzyme to RNA as in the p20 gel-shift experiments: these complexes presumably involve only the stronger RNA binding region (region 1 [16]) and allow the protein to pack onto the RNA to a density of one molecule per helical turn.

According to this model, activation of the enzyme requires bivalent dsRNA binding which becomes detectable at approximately 30 bp and is most stable when the duplex is at least 80 bp long. Correlating with the formation of the most stable complexes is a shift in their mobility in the gel retardation assay. The predominant complex formed with dsRNA with a length of ≥85 bp is band III, which moves faster than the predominant complex formed with shorter dsRNA (band I). We considered the possibility that longer duplexes might be able to bind more DAI molecules than shorter duplexes, but because DAI is a basic protein (pI 8.6), it is unlikely that the acceleration in gel mobility that occurs between 67 and 85 bp with the shift from complex I to complex III is due to the binding of a second DAI molecule to a DAI-dsRNA complex. Therefore, we argue that the faster migration is probably due to a conformational change in the dsRNA or the DAI-dsRNA complex which leads to compaction and increased electrophoretic mobility. Compaction could result from relief of the distortion in DAI that occurs when the two binding elements can interact with optimally spaced sites on dsRNA. Alternatively, it could be accomplished if the RNA were bent or wrapped around the enzyme once it had filled the entire site. If this explanation is correct, it seems that the duplex must be continuous since an elevated concentration of 40-bp molecules does not have the same effect on binding or activation as an 80-bp duplex. The minor complexes, II and IV, seem to be related to complexes I and III, respectively, but display increased sensitivity to competition with tRNA. They are unlikely to represent the addition of a second molecule of DAI to a DAI-dsRNA complex because of the large retardation effect that this would be expected to have on electrophoretic mobility and there are few clues as to their structure or significance at present.

The proposal that DAI contains a single bipartite RNA-binding site provides an alternative to the two previous models for DAI activation, neither of which is readily compatible with the results presented here. The gel-shift data could be interpreted in terms of the model in which DAI possesses two distinct sites for dsRNA binding (15, 37), a high-affinity site for activation and a low-affinity inhibitory site, if it were supposed that duplexes with sizes of ≤67 bp bind at the inhibitory site whereas longer duplexes bind at the activating site. The resultant complexes could have significantly different mobilities. However, the apparent affinities for these duplexes are not greatly different in the binding assays shown here, so the postulate that the activation site is of much higher affinity than the inhibitory site is not satisfied. Moreover, 40- to 67-bp duplexes have significant ability to activate the enzyme. The data could also be interpreted in terms of the model that DAI is activated when two molecules bind to a single molecule of dsRNA (37, 49). On this basis, 80 bp would be the length of duplex required to span the RNA-binding sites of two DAI monomers. Each monomer would interact with 30 to 40 bp of dsRNA, and the complex would be stabilized by cooperative interactions between the protein molecules. The monotonous increase in binding efficiency with a chain length between 30 and 85 bp argues against this model, as does our failure to obtain protection of shorter RNA fragments at high ratios of DAI to dsRNA. Also, with this model, it is difficult to explain the multiplicity of complexes observed in band-shift experiments: in particular, longer duplexes or higher DAI concentrations would be expected to give rise to slower complexes, contrary to observations.

In summary, the data presented here suggest that DAI interacts with as little as 11 bp (one helical turn) of dsRNA, but activation is associated with the formation of a stable DAI-dsRNA complex. The formation of such a complex requires at least 30 bp of duplex (about three turns) and probably takes place when both of the enzyme's RNA-binding motifs are engaged with the ligand. Complex formation is optimal with dsRNA containing at least 80 bp (seven

to eight turns) and is apparently accompanied by a conformational change in the complex. We speculate that the bivalent interaction with dsRNA or the conformational change itself is critical for enzyme autophosphorylation and activation. With this model, short dsRNAs would be expected to block activation because they can interact with only one RNA-binding motif. Specific inhibitors of DAI activation, such as VA RNA, may also bind to one motif, or alternatively they may bind to both motifs but in such a way as to interfere with the conformation of the enzyme. Similarly, long dsRNAs that activate the enzyme at low concentrations might block activation at high concentrations because the two binding motifs form complexes with separate RNA duplexes, thereby precluding the requisite conformational change. While this is a satisfying explanation, there are alternatives which also fit the available facts. For example, since autophosphorylation appears to be intermolecular (29, 37), it is also possible that DAI serves as a phosphate acceptor only when it is not bound to dsRNA, a situation which would obtain at low or moderate concentrations of dsRNA. Consistent with this view, a truncated form of DAI that lacks the RNA binding site can still be phosphorylated by intact DAI (unpublished data). It is clear that further investigation will be required to establish the nature of the coupling between dsRNA binding and kinase activation: such studies are in progress.

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REFERENCES

1. **Baglioni, C., and P. A. Maroney.** 1981. Inhibition of double-stranded ribonucleic acid activated protein kinase and 2',5'-oligo(adenylic acid) polymerase by ethidium bromide. Biochemistry **20**:758–762.
2. **Baglioni, C., M. A. Minks, and E. DeClercq.** 1981. Structural requirements of polynucleotides for the activation of (2'-5')A_n polymerase and protein kinase. Nucleic Acids Res. **9**:4939–4950.
3. **Berry, M. J., G. S. Knutson, S. R. Laskey, S. M. Munemitsu, and C. E. Samuel.** 1985. Purification and substrate specificities of the double-stranded RNA-dependent protein kinase from untreated and interferon-treated mouse fibroblasts. J. Biol. Chem. **260**:11240–11247.
4. **Bhat, R. A., and B. Thimmappaya.** 1985. Construction and analysis of additional adenovirus substitution mutants confirm the complementation of VAI RNA function by two small RNAs encoded by Epstein-Barr virus. J. Virol. **56**:750–756.
5. **Bischoff, J. R., and C. E. Samuel.** 1989. Mechanism of interferon action. Activation of the human P1/eIF-2α protein kinase by individual reovirus s-class mRNAs: s1 mRNA is a potent activator relative to s4 mRNA. Virology **172**:106–115.
6. **Chong, K. L., L. Feng, K. Schappert, E. Meurs, T. F. Donahue, J. D. Friesen, A. G. Hovenessian, and B. R. G. Williams.** 1992. Human p68 kinase exhibits growth suppression in yeast and homology to the translational regulator *GCN2*. EMBO J. **11**: 1553–1562.
7. **Clarke, P. A., N. A. Sharp, and M. J. Clemens.** 1990. Translational control by the Epstein-Barr virus small RNA EBER-1. Reversal of the double-stranded RNA-induced inhibition of protein synthesis in reticulocyte lysates. Eur. J. Biochem. **193**:635–641.
8. **deBenedetti, A., and C. Baglioni.** 1984. Inhibition of mRNA binding to ribosomes by localized activation of dsRNA-dependent protein kinase. Nature (London) **311**:79–81.
9. **Dever, T. E., L. Feng, R. C. Wek, A. M. Cigan, T. F. Donahue, and A. G. Hinnebusch.** 1992. Phosphorylation of initiation factor 2α by protein kinase GCN2 mediates gene-specific translational control of *GCN4* in yeast. Cell **68**:585–596.
10. **Dubois, M.-F., J. Galabru, P. Lebon, B. Safer, and A. G. Hovanessian.** 1989. Reduced activity of the interferon-induced double-stranded RNA-dependent protein kinase during a heat shock stress. J. Biol. Chem. **264**:12165–12171.
11. **Farrell, P. J., K. Balkow, T. Hunt, and R. J. Jackson.** 1977. Phosphorylation of initiation factor eIF-2 and the control of reticulocyte protein synthesis. Cell **11**:187–200.
12. **Feng, G.-S., K. Chong, A. Kumar, and B. R. G. Williams.** 1992. Identification of double-stranded RNA-binding domains in the interferon-induced double-stranded RNA-activated p68 kinase. Proc. Natl. Acad. Sci. USA **89**:5447–5451.
13. **Galabru, J., and A. Hovanessian.** 1985. Two interferon-induced proteins are involved in the protein kinase complex dependent on double-stranded RNA. Cell **43**:685–694.
14. **Galabru, J., and A. Hovanessian.** 1987. Autophosphorylation of the protein kinase dependent on double-stranded RNA. J. Biol. Chem. **262**:15538–15544.
15. **Galabru, J., M. G. Katze, N. Robert, and A. G. Hovanessian.** 1989. The binding of double-stranded RNA and adenovirus VAI RNA to the interferon-induced protein kinase. Eur. J. Biochem. **178**:581–589.
16. **Green, S. R., and M. B. Mathews.** Submitted for publication.
17. **Grodberg, J., and J. J. Dunn.** 1988. *ompT* encodes the *Escherichia coli* outer membrane protease that cleaves T7 RNA polymerase during purification. J. Bacteriol. **170**:1245–1253.
18. **Gunnery, S., A. P. Rice, H. D. Robertson, and M. B. Mathews.** 1990. Tat-responsive region RNA of human immunodeficiency virus 1 can prevent activation of the double-stranded-RNA-activated protein kinase. Proc. Natl. Acad. Sci. USA **87**:8687–8691.
19. **Hershey, J. W. B.** 1989. Protein phosphorylation controls translation rates. J. Biol. Chem. **264**:20823–20826.
20. **Hershey, J. W. B.** 1991. Translational control in mammalian cells. Annu. Rev. Biochem. **60**:717–755.
21. **Hovanessian, A. G.** 1989. The double stranded RNA-activated protein kinase induced by interferon: dsRNA-PK. J. Interferon Res. **9**:641–647.
22. **Hunter, T., T. Hunt, R. J. Jackson, and H. D. Robertson.** 1975. The characteristics of inhibition of protein synthesis by double-stranded ribonucleic acid in reticulocyte lysates. J. Biol. Chem. **250**:409–417.
23. **Judware, R., and R. Petryshyn.** 1991. Partial characterization of a cellular factor that regulates the double-stranded RNA-dependent eIF-2α kinase in 3T3-F442A fibroblasts. Mol. Cell. Biol. **11**:3259–3267.
24. **Katze, M. G., B. M. Detjen, B. Safer, and R. M. Krug.** 1986. Translational control by influenza virus: suppression of the kinase that phosphorylates the alpha subunit of initiation factor eIF-2 and selective translation of influenza viral messenger RNAs. Mol. Cell. Biol. **6**:1741–1750.
25. **Katze, M. G., M. Wambach, M.-L. Wong, M. Garfinkel, E. Meurs, K. Chong, B. R. G. Williams, A. G. Hovanessian, and G. N. Barber.** 1991. Functional expression and RNA binding analysis of the interferon-induced, double-stranded RNA-activated, 68,000-M_r protein kinase in a cell-free system. Mol. Cell. Biol. **11**:5497–5505.
26. **Kaufman, R. J., and P. Murtha.** 1987. Translational control mediated by eukaryotic initiation factor-2 is restricted to specific mRNAs in transfected cells. Mol. Cell. Biol. **7**:1568–1571.
27. **Kimchi, A., A. Zilberstein, A. Schmidt, L. Shulman, and M. Revel.** 1979. The interferon-induced protein kinase PK-i from mouse L cells. J. Biol. Chem. **254**:9846–9853.
28. **Kitajewski, J., R. J. Schneider, B. Safer, S. M. Munemitsu, C. E. Samuel, B. Thimmappaya, and T. Shenk.** 1986. Adenovirus VAI RNA antagonizes the antiviral action of the interferon by preventing activation of the interferon-induced eIF-2α kinase. Cell **45**:195–200.

29. **Kostura, M., and M. B. Mathews.** 1989. Purification and activation of the double-stranded RNA-dependent eIF-2 kinase DAI. Mol. Cell. Biol. **9**:1576–1586.
30. **Lasky, S. R., B. L. Jacobs, and C. E. Samuel.** 1982. Characterization of the sites of phosphorylation in the interferon-induced phosphoprotein P1 from mouse fibroblasts: evidence for two forms of P1. J. Biol. Chem. **257**:11087–11093.
31. **Laurent, A. G., B. Krust, J. Galabru, J. Svab, and A. G. Hovanessian.** 1985. Monoclonal antibodies to an interferon-induced M_r 68,000 protein and their use for the detection of double-stranded RNA-dependent protein kinase in human cells. Proc. Natl. Acad. Sci. USA **82**:4341–4345.
32. **Lebleu, B., G. C. Sen, S. Shaila, B. Cabrer, and P. Lengyel.** 1976. Interferon, double-stranded RNA, and protein phosphorylation. Proc. Natl. Acad. Sci. USA **73**:3107–3111.
33. **Lenz, J. R., and C. Baglioni.** 1978. Inhibition of protein synthesis by double-stranded RNA and phosphorylation of initiation factor, eIF-2. J. Biol. Chem. **253**:4219–4223.
34. **Levin, D., and I. M. London.** 1978. Regulation of protein synthesis: activation by double-stranded RNA of a protein kinase that phosphorylates eukaryotic initiation factor 2. Proc. Natl. Acad. Sci. USA **75**:1121–1125.
35. **Levin, D. H., R. Petryshyn, and I. M. London.** 1980. Characterization of double-stranded-RNA-activated kinase that phosphorylates α subunit of eukaryotic initiation factor 2 (eIF-2α) in reticulocyte lysates. Proc. Natl. Acad. Sci. USA **77**:832–836.
36. **Mathews, M. B.** 1991. Control of translation in adenovirus infected cells. Enzyme **44**:250–264.
37. **Mathews, M. B., S. Gunnery, L. Manche, K. H. Mellits, and T. Pe'ery.** 1990. Control of protein synthesis by RNA regulators. NATO ASI (Adv Sci Inst) Ser H Cell Biol. **49**:377–388.
38. **Mathews, M. B., and T. Shenk.** 1991. Adenovirus virus-associated RNA and translational control. J. Virol. **65**:5657–5662.
39. **McCormack, S. J., D. C. Thomis, and C. E. Samuel.** 1992. Mechanism of interferon action: identification of a RNA binding domain within the N-terminal region of the human RNA-dependent P1/eIF-2α protein kinase. Virology **188**:47–56.
40. **Mellits, K. H., M. Kostura, and M. B. Mathews.** 1990. Interaction of adenovirus VA RNA_I with the protein kinase DAI: non-equivalence of binding and function. Cell **61**:843–852.
41. **Mellits, K. H., and M. B. Mathews.** 1988. Effects of mutations in stem and loop regions on the structure and function of adenovirus VA RNA_I. EMBO J. **7**:2849–2859.
42. **Mellits, K. H., T. Pe'ery, L. Manche, H. D. Robertson, and M. B. Mathews.** 1990. Removal of double-stranded contaminants from RNA transcripts: synthesis of adenovirus VA RNA_I from a T7 vector. Nucleic Acids Res. **18**:5401–5406.
43. **Meurs, E., K. Chong, J. Galabru, N. S. B. Thomas, I. M. Kerr, B. R. G. Williams, and A. G. Hovanessian.** 1990. Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. Cell **62**: 379–390.
44. **Minks, M. A., D. K. West, S. Benvin, and C. Baglioni.** 1979. Structural requirements of double-stranded RNA for the activation of 2′,5′-oligo(A) polymerase and protein kinase of interferon-treated HeLa cells. J. Biol. Chem. **254**:10180–10183.
45. **Minks, M. A., D. K. West, S. Benvin, J. J. Greene, P. O. P. Ts'o, and C. Baglioni.** 1980. Activation of 2′,5′-oligo(A) polymerase and protein kinase of interferon-treated HeLa cells by 2′-O-methylated poly (inosinic acid) · poly (cytidylic acid). J. Biol. Chem. **255**:6403–6407.
46. **Ochoa, S.** 1983. Regulation of protein synthesis initiation in eukaryotes. Arch. Biochem. Biophys. **223**:325–349.
47. **O'Malley, R. P., R. F. Duncan, J. W. B. Hershey, and M. B. Mathews.** 1989. Modification of protein synthesis initiation factors and shut-off of host protein synthesis in adenovirus-infected cells. Virology **168**:112–118.
48. **O'Malley, R. P., T. M. Mariano, J. Siekierka, and M. B. Mathews.** 1986. A mechanism for the control of protein synthesis by adenovirus VA RNA_I. Cell **44**:391–400.
49. **O'Malley, R. P., T. M. Mariano, J. Siekierka, W. C. Merrick, P. A. Reichel, and M. B. Mathews.** 1986. The control of protein synthesis by adenovirus VA RNA. Cancer Cells **4**:291–301.
50. **Patel, R. C., and G. C. Sen.** 1992. Identification of the double-stranded RNA-binding domain of the human interferon-inducible protein kinase. J. Biol. Chem. **267**:7671–7676.
51. **Pestka, S., J. A. Langer, K. C. Zoon, and C. E. Samuel.** 1987. Interferons and their actions. Annu. Rev. Biochem. **56**:727–777.
52. **Petryshyn, R., J.-J. Chen, and I. M. London.** 1984. Growth-related expression of a double-stranded RNA-dependent protein kinase in 3T3 cells. J. Biol. Chem. **259**:14736–14742.
53. **Rice, A. R., M. Kostura, and M. B. Mathews.** 1989. Identification of a 90 KD polypeptide which associates with adenovirus VA RNA_I and is phosphorylated by the double-stranded RNA dependent protein kinase. J. Biol. Chem. **264**:20632–20637.
54. **Samuel, C. E.** 1991. Antiviral actions of interferon: interferon-regulated cellular proteins and their surprisingly selective antiviral activities. Virology **183**:1–11.
55. **Schmedt, C., S. R. Green, and M. B. Mathews.** Unpublished data.
56. **Schneider, R. J., and T. Shenk.** 1987. Impact of virus infection on host cell protein synthesis. Annu. Rev. Biochem. **56**:317–332.
57. **Schweitz, H., and J. P. Ebel.** 1971. A study of the mechanism of action of E. coli ribonuclease III. Biochimie **53**:585–593.
58. **Sen, G. C., H. Taira, and P. Lengyel.** 1978. Interferon, double-stranded RNA, and protein phosphorylation. J. Biol. Chem. **253**:5915–5921.
59. **Sonenberg, N.** 1990. Measures and countermeasures in the modulation of initiation factor activities by viruses. New Biol. **2**:402–409.
60. **Svensson, C., and G. Akusjärvi.** 1985. Adenovirus VA RNA_I mediates a translational stimulation which is not restricted to the viral mRNAs. EMBO J. **4**:957–964.
61. **Tiwari, R. K., J. Kusari, R. Kumar, and G. C. Sen.** 1988. Gene induction by interferons and double-stranded RNA: selective inhibition by 2-aminopurine. Mol. Cell. Biol. **8**:4289–4294.
62. **Torrence, P. F., M. I. Johnston, D. A. Epstein, H. Jacobsen, and R. M. Friedman.** 1981. Activation of human and mouse 2-5A synthetases and mouse protein P_1 kinase by nucleic acids. FEBS Lett. **130**:291–296.
63. **Zilberstein, A., A. Kimchi, A. Schmidt, and M. Revel.** 1978. Isolation of two interferon-induced translational inhibitors: a protein kinase and an oligo-isoadenylate synthetase. Proc. Natl. Acad. Sci. USA **75**:4734–4738.
64. **Zinn, K., A. Keller, L.-A. Whitemore, and T. Maniatis.** 1988. 2-Aminopurine selectively inhibits the induction of β-interferon, c-*fos*, and c-*myc* gene expression. Science **240**:210–213.

ATTACHMENT C

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Structural Requirements of Double-stranded RNA for the Activation of 2',5'-Oligo(A) Polymerase and Protein Kinase of Interferon-treated HeLa Cells*

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Addition of double-stranded RNA (dsRNA) to extracts of interferon-treated HeLa cells results in the synthesis of 2',5'-oligo(A) from ATP and in the phosphorylation of a ribosome-associated protein of M_r = 72,000. Previously described assays were used to investigate the structural requirements of dsRNA for the activation of these two enzymatic activities. Poly(CG) with different ratios of C/G was synthesized with polynucleotide phosphorylase. These polynucleotides were either annealed with poly(I) to form mismatched dsRNA or digested with ribonuclease T_1 to produce smaller polynucleotides. Polymers with an average of one mismatch every eight nucleotides failed to activate the 2',5'-oligo(A) polymerase and protein kinase, whereas polymers with a mismatch every 45 nucleotides were fully active. The polynucleotides obtained by T_1 digestion of poly(CG) were fractionated by gel filtration into discrete size polymers. These sized polynucleotides were annealed with high molecular weight poly(I) and assayed for activation of 2',5'-oligo(A) polymerase and protein kinase. These enzymes could not be activated by dsRNA containing poly(C) shorter than 30 nucleotides. Maximal activation was obtained with dsRNA containing poly(C) longer than 65 to 80 nucleotides. A similar size requirement for activation was observed with dsRNA formed with poly(A) and poly(U) of known length. These results indicate that a relatively long stretch of base pairs, uninterrupted by either a mismatch or a discontinuity in one of the complementary strands, is required for the activation of the two enzymes studied. These structural characteristics are similar to those previously shown to be required for the induction of interferon by dsRNA.

Natural and synthetic dsRNAs[1] are among the most potent interferon inducers (1). Furthermore, dsRNA is also a potent inhibitor of protein synthesis in extracts of interferon-treated cells (2). This inhibition is due to elevated levels of a protein kinase and an oligonucleotide polymerase which require dsRNA, ATP, and Mg^{2+} for activity (see Ref. 3). Activation of the protein kinase by dsRNA results in the phosphorylation of a polypeptide of about M_r = 70,000 and of the α subunit of initiation factor eIF-2 (3, 4). The oligonucleotide polymerase, designated 2',5'-oligo(A) polymerase (3), synthesizes from ATP a series of oligonucleotides containing the unusual 2',5'-phosphodiester linkage (5). The 2',5'-oligo(A) is not itself inhibitory to protein synthesis, but activates an endonuclease present in both control and interferon-treated cells and inhibition of protein synthesis occurs via mRNA degradation (6, 7).

Relatively little is known about the molecular features of dsRNA which are required for the activation of the protein kinase and 2',5'-oligo(A) polymerase. RNA/DNA hybrids and the triple-stranded polymer poly(A)·poly(U)·poly(U) do not activate the latter enzyme (8). In contrast, there is vast literature on the molecular features of dsRNA which are relevant for the induction of interferon (1). For example, the presence of mismatched nucleotides in synthetic dsRNA causes a loss of activity (9) and the dsRNA must be longer than approximately 50 base pairs to induce interferon (10).

We report here the results of an investigation of the effect of mismatched nucleotides and of polymer size on the activation of 2',5'-oligo(A) polymerase and protein kinase by dsRNA. Interestingly, the structural features of dsRNA relevant for the activation of these enzymes are similar to those reported in the literature for the induction of interferon.

EXPERIMENTAL PROCEDURES

Chemicals—Radiochemicals were purchased from New England Nuclear; sized poly(A) and poly(U) from Miles; polynucleotide phosphorylase from P-L Biochemicals; nuclease T_1 from Sigma.

Cells, Extracts, and Assays—HeLa cells grown in suspension culture were treated with 100 reference units/ml of human fibroblast interferon (3×10^6 units/mg) for 17 h prior to harvest. Interferon was obtained from the Interferon Working Group, National Cancer Institute, NIH. Extracts were prepared from these cells as previously described (8). Ribosomes were isolated by centrifugation and resuspended as described (11). Protein kinase activity was determined in assays containing 0.1 mM [γ-^{32}P]ATP (1.3 Ci/mol) and 30 to 40 μg of ribosomes in a final volume of 30 μl. Incubation was at 30°C for 7 min prior to fractionation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11). Autoradiographs of the gels were scanned at 560 nm, and the area under the peak corresponding to a polypeptide of M_r = 72,000 was measured as previously described (12). Assays for the synthesis of 2',5'-oligo(A) contained unless otherwise indicated 5 μl of cell extract (about 10 mg protein/ml), 0.12 M KOAc, 25 mM $Mg(OAc)_2$, 20 mM Hepes/KOH, pH 7.4, 5 mM [^{3}H]ATP (1.6 Ci/mol), 4 mM fructose 1,6-bis-phosphate, 1 mM dithiothreitol, and the indicated amount of dsRNA in a final incubation volume of 25 μl. Incubation was at 30°C for 60 min and reactions were terminated by heating to 95°C for 3 min. The 2',5'-oligo(A) formed was determined by chromatography on DEAE-cellulose as previously described (8).

Preparation of CG Copolymers, Sized Polynucleotides, and Double-stranded RNA—Ribonucleotide diphosphates were polymerized with *Micrococcus lysodeikticus* polynucleotide phosphorylase (2 mg/ml) at 37°C for 2 h as described (9). Reactions contained CDP and [^{3}H]GDP in molar ratios of 10:1 to 200:1. Polynucleotides were extracted with phenol and dialyzed exhaustively (9). The C and G

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[1] The abbreviations used are: dsRNA, double-stranded RNA; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

content of polynucleotides was calculated from the A_{271}/counts per min ratio of dialyzed material digested overnight with 0.3 N KOH by comparison of this ratio with that of the unpolymerized starting material. Sized polynucleotides were prepared from poly(CG) by 3-h digestion at 37°C with 0.5 to 1 unit of ribonuclease T_1 per A_{271} unit. The digestion products were applied to columns of Sephadex G-200, G-150, or G-50, depending on the expected size of the fragments generated. The size of the polynucleotides in the eluted fractions was estimated from the A_{268}/counts per min ratio. Correction was made for variation of extinction coefficient with chain length by assuming a negligible contribution to absorbance by the G residue present in the polynucleotides and taking absorbance values for oligo(C) from Adler *et al.* (13). Double-stranded RNA was formed by heating equimolar nucleotide amounts of complementary RNA species to 70°C in a buffer containing 0.1 M KOAc and 20 mM Hepes/KOH, pH 7.4, and cooling to 30°C. Poly(I) of M_r > 100,000 ($s_{20,w}$ = 9.4) was used.

RESULTS

Effect of Base Mismatching on the Activation of 2',5'-Oligo(A) Polymerase and Protein Kinase—Polyribonucleotides containing different ratios of C to G were synthesized with polynucleotide phosphorylase. These polynucleotides were annealed with poly(I) to form dsRNA containing mismatched bases, since G cannot form a normal Watson-Crick base pair with I. The relative mismatching of these dsRNAs is inversely proportional to the C/G ratio. The dsRNAs are designated by the C/G ratio of the poly(CG) strand.

Polymers with a C/G ratio of 7 do not promote synthesis of 2',5'-oligo(A) or phosphorylation of the M_r = 72,000 polypeptide (Figs. 1; 2, *A* and *D*). Polymers with a C/G ratio of 15 are partially active in both assays and polymers with a ratio of 45 or higher are fully active.

The effect of increasing concentrations of polymer on the synthesis of 2',5'-oligo(A) was next investigated. Polymers with a C/G ratio of 45 show maximum activity at 5 μg/ml, whereas a polymer with a ratio of 7 shows less than 5% of this activity at 20 μg/ml (data not shown). The activity of polymers of different C/G ratio cannot be explained by differential degradation of these polynucleotides. No degradation to acid-soluble material was detected when polymers of C/G ratio of 7 and 15 were incubated for 90 min under the conditions of our assays (data not shown). The simplest interpretation of the above results is that a minimum length of perfectly matched I·C base pairs is necessary for activation of 2',5'-oligo(A) polymerase and protein kinase.

Size Requirements for Activation of 2',5'-Oligo(A) Polymerase and Protein Kinase—Polynucleotides of different C/G ratios were digested with ribonuclease T_1 to yield a series of poly(C) fragments terminating in G (9). These fragments were fractionated by gel filtration and their size determined as described under "Experimental Procedures." In order to generate a full series of sized fragments, polynucleotides with C/G ratios of 200, 45, and 20 were digested and fractionated in this way. Sized poly(C) fragments ranging in average length between 10 and 380 nucleotides were thus obtained. These sized polynucleotides were annealed with equimolar nucleotide amounts of poly(I). The dsRNAs formed are designated as $I_n \cdot C_x$, where x indicates the average number of C residues of a sized polynucleotide terminating in G. The dsRNAs were assayed for activation of 2',5'-oligo(A) polymerase and protein kinase (Figs. 1 and 2, *B* and *E*). Both enzymes were activated only by dsRNAs containing poly(C) longer than 35 nucleotides, with maximal activity being observed with $I_n \cdot C_{65}$ for synthesis of 2',5'-oligo(A) and with $I_n \cdot C_{80}$ in the kinase assay.

Partial activation of 2',5'-oligo(A) polymerase and protein kinase by dsRNAs containing poly(C) 35 to 50 nucleotides long could be due to contamination of the sized polynucleotides with poly(C) of greater length. To test this possibility, we determined the effect of increasing concentrations of dsRNA on the activation of 2',5'-oligo(A) polymerase (Fig. 3*A*). The polymer $I_n \cdot C_{32}$ was inactive below 10 μg/ml, but at 20 μg/ml had 3% of the activity of $I_n \cdot C_{55}$. We cannot distinguish, however, between a 3% contamination of C_{32} with longer poly(C) and a marginal activation of the enzyme by $I_n \cdot C_{32}$.

In the experiments described above, the 2',5'-oligo(A) polymerase assay was carried out at 20 mM $Mg(OAc)_2$ which gives optimal synthesis of 2',5'-oligo(A) with HeLa cell extracts (8). When 2',5'-oligo(A) synthesis was assayed at the same $Mg(OAc)_2$ used in the kinase assay (2 mM), an increase in the size of dsRNA required for activation was observed. The polymer $I_n \cdot C_{37}$ showed little activity and $I_n \cdot C_{32}$ was

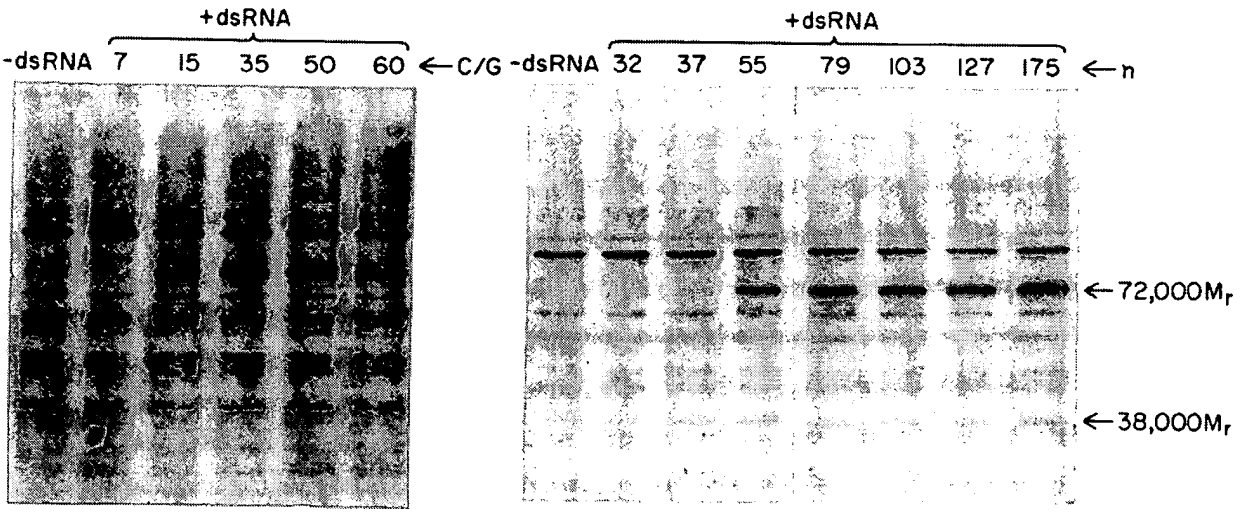

FIG. 1. **Phosphorylation of the M_r = 72,000 polypeptide by the protein kinase activated with poly(I)·poly(CG) (*left*) or by poly(I) annealed to sized poly(C) (*right*).** Ribosomes from interferon-treated HeLa cells were incubated with or without 0.5 (*left*) or 0.1 (*right*) μg/ml of dsRNA for 7 min at 30°C and analyzed by gel electrophoresis and autoradiography as described under "Experimental Procedures." The tracks show from *left* to *right*: an incubation without added dsRNA; incubations with added poly(I)·poly(CG), with the ratio C/G of the polymer indicated for each track; an incubation without added dsRNA; incubations with added poly(I) annealed to poly(C) of the length indicated for each track (*n*). The position of the M_r = 72,000 and 38,000 polypeptides is indicated on the *right*.

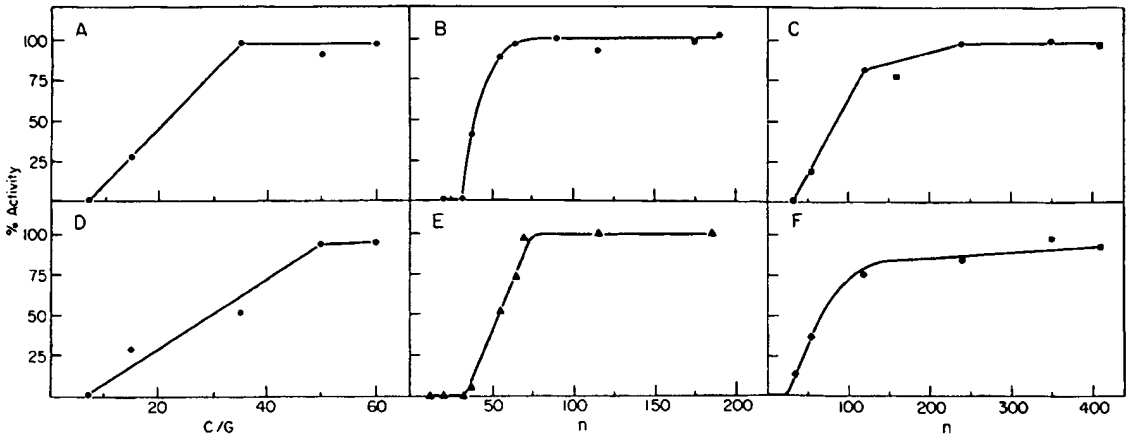

FIG. 2. **Synthesis of 2',5'-oligo(A) (*A*, *B*, and *C*) and phosphorylation of the M_r = 72,000 polypeptide (*D*, *E*, and *F*) promoted by poly(I)·poly(CG) with different ratios of C/G (*A* and *D*), by poly(I) annealed to sized poly(C) (*B* and *E*), and by sized poly(A) annealed to sized poly(U) (*C* and *F*).** Preparation of polymers and assays for 2',5'-oligo(A) polymerase and protein kinase are described under "Experimental Procedures." The polymers were tested at 10 μg/ml in the polymerase assay and at 0.5 (*D*) or 0.1 (*E* and *F*) μg/ml in the kinase assay. The activity is expressed as a percentage of that obtained with poly(I)·poly(C). With this polymer, 33 nmol of ATP were converted to 2',5'-oligo(A) from an input of 125 nmol of ATP per reaction. In the kinase assay, phosphorylation was determined by scanning autoradiographs of gels, like those shown in Fig. 1, and measuring the area under the M_r = 72,000 band. Phosphorylation observed with different polymers is shown as a percentage of that obtained with poly(I)·poly(C). On the *abscissa* is indicated the C/G ratio of the polymers tested (*left panels*), the length in nucleotides of the poly(C) annealed to poly(I) (*middle panels*), and the length of the shorter polynucleotide used to form dsRNA with sized poly(A) and poly(U) (*right panels*). Different symbols designate in these latter panels the longer complementary polynucleotides annealed: (●) A_{410}, (◆) U_{120}, and (■) U_{500}.

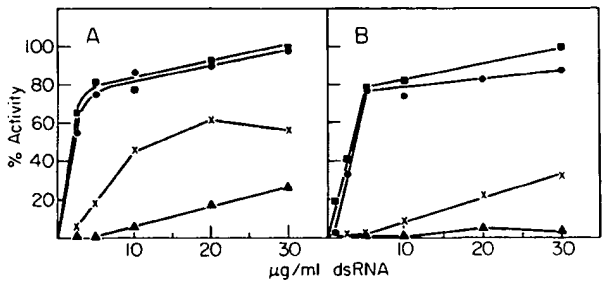

FIG. 3. **Activation of 2',5'-oligo(A) polymerase by different concentrations of poly(I) annealed to sized poly(C) with (*A*) 20 mM and (*B*) 2 mM $Mg(OAc)_2$.** Increasing concentration of poly(I)·C_{32} (▲——▲), poly(I)·C_{37} (×——×), poly(I)·C_{55} (●——●), and poly(I)·C_{380} (■——■) were added to standard incubation mixtures. With 30 μg/ml of poly(I)·poly(C) and 20 mM $Mg(OAc)_2$, 40 nmol of ATP were converted to 2',5'-oligo(A) from an input of 125 nmol, whereas 20 nmol were converted with 2 mM $Mg(OAc)_2$. Each reaction contained also 5 mM $Mg(OAc)_2$ added in equimolar amount with ATP (see "Experimental Procedures"). The synthesis of 2',5'-oligo(A) is expressed as a percentage of that obtained with 30 μg/ml of poly(I)·poly(C).

practically inactive even at 30 μg/ml (Fig. 3*B*), whereas $I_n \cdot C_{55}$ remained fully active.

The failure of dsRNAs containing short poly(C) to activate either the 2',5'-oligo(A) polymerase or the protein kinase may be due to low affinity of the enzymes for these polymers. Alternatively, these dsRNAs may bind the enzymes but may not function as activators. This dsRNA/enzyme interaction was studied by competition experiments, in which an active dsRNA was assayed in the presence of increasing amounts of inactive dsRNA. No competition between 2.5 μg/ml of active dsRNA and 30 μg/ml of inactive dsRNA was detected in the 2',5'-oligo(A) polymerase assays (data not shown). The results of experiments testing competition in the protein kinase assay were more difficult to interpret. Activation of the kinase occurs only within a range of dsRNA concentrations, and high dsRNA levels are inhibitory (14). Addition of a 10-fold excess of $I_n \cdot C_{30}$ had no effect on kinase activation by 0.1 μg/ml of $I_n \cdot C_{130}$, whereas a 100-fold excess significantly impaired kinase activation. The concentration of dsRNA was raised in this case to 10 μg/ml. Both short and long polymers were inactive at this dsRNA concentration (Ref. 11 and data not shown). It seems possible, therefore, that inactive dsRNAs may prevent activation of the kinase by raising the dsRNA concentration to the inhibitory range.

Sized poly(A) and poly(U) were similarly tested upon annealing with equimolar nucleotide amounts of complementary strands. It was therefore possible to construct dsRNAs with two polynucleotides of known length. The dsRNAs containing A_{33} were inactive in the 2',5'-oligo(A) polymerase assay and only slightly active in the kinase assay (Fig. 2, *C* and *F*). The dsRNAs containing A_{54} were partially active in both assays, and those containing U_{120} annealed to longer poly(A) were fully active. The effect of the complementary chain length on the activation of 2',5'-oligo(A) polymerase was investigated in a systematic way (Table I). The activity of the dsRNAs was found to be dependent mainly on the length of the shorter polynucleotide. The length of the complementary polynucleotide is also important, however, as shown by the higher activity of A_{54} annealed with poly(U) of increasing length. A

TABLE I

Effect of varying poly(A) and poly(U) size on the synthesis of 2',5'-oligo(A)

Polynucleotide[a] (average size)		2',5'-Oligo(A) synthesized
A	U	
		nmol adenosine polymerized
33	120	0
54	120	0.7
54	240	1.4
54	500	6.3
160	120	14.3
160	350	19.1
160	500	25.5
410	240	32.5
410	500	31.7

[a] Equimolar nucleotide amounts of poly(A) and poly(U) were annealed and assayed at 20 μg/ml as described under "Experimental Procedures." Each reaction contained 125 nmol of [^{3}H]ATP.

possible explanation for this effect is that annealing of two relatively short polymers is likely to result in more discontinuities in complementary strands than when a shorter polymer is annealed with a longer one. More double-stranded sequences of the minimal length necessary for enzyme activation will be formed in this latter case.

DISCUSSION

We have studied the activation of two dsRNA-dependent enzymatic activities by preparations of poly(I)·poly(CG) containing different proportions of G residues (mismatched dsRNA). The results obtained clearly indicate that a minimum length of perfectly matched base pairs is necessary for the activation of these enzymes. This length can be roughly estimated by calculating the average frequency of C runs of different length in polymers with variable degree of mismatching. If a random distribution of C and G residues is assumed to occur in these polymers, the probability of finding a *C* run of length *n* is then given by $(C/(C+G))^n$ (where $(C/(C+G))$ is the relative proportion of *C* in the mismatched strand). Runs of 35 or more C's are 40-fold more frequent in a polymer with a C/G ratio of 35 than in a polymer with a ratio of 7. Experimentally, the former polymer was found to be at least 20-fold more active than the latter polymer in promoting synthesis of 2',5'-oligo(A).

A direct estimate of the dsRNA size requirement for activation of these enzymes was obtained by forming polymers with a high molecular weight poly(I) strand annealed to poly(C) of different length. Only dsRNA containing poly(C) longer than 65 to 80 nucleotides was fully active. These results were confirmed with poly(A) and poly(U) of known size annealed in different combinations. The structural requirements of dsRNA for activation of the 2',5'-oligo(A) polymerase and protein kinase are therefore similar. There are, however, some differences between these two enzymes. The 2',5'-oligo(A) polymerase is fully activated by poly(I)·poly(CG) with fewer mismatches than the kinase, and dsRNA of greater size is required for activation of this latter enzyme. These differences may in part be explained by the different Mg^{2+} concentration in the assays, since slightly larger dsRNA was required for maximal activation of 2',5'-oligo(A) polymerase at lower Mg^{2+} concentration. The activation of these two enzymes differs in another way: the kinase cannot be activated in the presence of high concentrations of dsRNA (14), whereas the 2',5'-oligo(A) polymerase is activated more effectively by high concentrations of dsRNA (8).

There are similarities between the structural requirements of dsRNA for interferon induction and those for activation of 2',5'-oligo(A) polymerase and protein kinase. These similarities have been previously noticed in studies assaying the inhibition of protein synthesis by dsRNA in reticulocyte lysates (15) and extracts of interferon-treated L cells (16), presumably due to the combined action of the protein kinase and 2',5'-oligo(A) polymerase/endonuclease system. A threshold molecular size of dsRNA corresponding to approximately 50 base pairs determines the interferon inducing activity of dsRNA (10). A similar size requirement is observed for the inhibition of protein synthesis in reticulocyte lysates by dsRNA (14). These observations agree with our findings that about 40 to 60 base pairs of dsRNA are required for the activation of 2',5'-oligo(A) polymerase and protein kinase under different assay conditions. Similarly, the ability of dsRNA to induce interferon decreases with increasing content of mismatched bases (9, 17).

Our results are directly comparable to those obtained by Carter *et al.* (9) with poly(I)·poly(C_{20}G). This polymer can partially activate the 2',5'-oligo(A) polymerase and protein kinase and has an intermediate interferon-inducing activity. Further work on the interferon-inducing activity of the other polymers used in our studies could possibly provide additional evidence for this correlation.

Some differences between the interferon-inducing activity and the inhibitory effect on protein synthesis of natural and synthetic dsRNA have been previously described (15, 16). Certain synthetic dsRNAs containing modified nucleotides are extremely efficient interferon inducers but do not significantly inhibit protein synthesis in reticulocyte lysates (15) or in extracts of interferon-treated L cells (16). Assays of these dsRNAs for activation of the 2',5'-oligo(A) polymerase and protein kinase will establish how closely the interferon-inducing activity of dsRNA is correlated with its ability to activate these enzymes. It is tempting to speculate that a dsRNA-dependent interferon-induced enzyme is part of the cellular recognition system for dsRNA. Both 2',5'-oligo(A) polymerase and protein kinase are present at a basal level in all mammalian and avian cells studied thus far (3). A dsRNA formed by a replicating virus or administered to cells may interact with these enzymes, which are known to bind dsRNA (18), and activate synthesis of 2',5'-oligo(A). This compound may in turn have some biological role in the induction of interferon synthesis. Further studies are necessary to provide some experimental support for this working hypothesis.

REFERENCES

1. Torrence, P. F., and De Clercq, E. (1977) *Pharmacol. Ther. Part A* **2**, 1-88
2. Kerr, I. M., Brown, R. E., and Ball, L. A. (1974) *Nature* **250**, 57-59
3. Baglioni, C. (1979) *Cell* **17**, 255-264
4. Sen, G. C., Taira, H., and Lengyel, P. (1978) *J. Biol. Chem.* **253**, 5915-5921
5. Kerr, I. M., and Brown, R. E. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 256-260
6. Baglioni, C., Minks, M. A., and Maroney, P. A. (1978) *Nature* **273**, 684-687
7. Clemens, M. J., and Williams, B. R. G. (1978) *Cell* **13**, 565-572
8. Minks, M. A., Benvin, S., Maroney, P. A., and Baglioni, C. (1979) *J. Biol. Chem.* **254**, 5058-5064
9. Carter, W. A., Pitha, P. M., Marshall, L. W., Tazawa, I., Tazawa, S., and Ts'o, P. O. P. (1972) *J. Mol. Biol.* **70**, 567-587
10. DeClercq, E. (1974) *Top. Curr. Chem.* **52**, 173-208
11. Lenz, J. R., and Baglioni, C. (1978) *J. Biol. Chem.* **253**, 4219-4223
12. West, D. K., and Baglioni, C. (1979) *Eur. J. Biochem.*, in press
13. Adler, A., Grossman, L., and Fosman, G. D. (1967) *Proc. Natl. Acad. Sci. U. S. A.* **57**, 423-430
14. Hunter, T., Hunt, T., Jackson, R. J., and Robertson, H. D. (1975) *J. Biol. Chem.* **250**, 409-417
15. Content, J., Lebleu, B., and De Clercq, E. (1978) *Biochemistry* **17**, 88-93
16. Torrence, P. F., and Friedman, R. M. (1979) *J. Biol. Chem.* **254**, 1259-1267
17. Matsuda, S., Kida, M., Shirafugi, H., Yoneda, M., and Yaoi, H. (1971) *Arch. Gesamte Virusforsch.* **34**, 105-118
18. Hovanessian, A. G., and Kerr, I. M. (1979) *Eur. J. Biochem.* **93**, 515-526

ATTACHMENT D

Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems

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Short interfering RNAs (siRNAs) are double-stranded RNAs of ≈21–25 nucleotides that have been shown to function as key intermediaries in triggering sequence-specific RNA degradation during posttranscriptional gene silencing in plants and RNA interference in invertebrates. siRNAs have a characteristic structure, with 5′-phosphate/3′-hydroxyl ends and a 2-base 3′ overhang on each strand of the duplex. In this study, we present data that synthetic siRNAs can induce gene-specific inhibition of expression in *Caenorhabditis elegans* and in cell lines from humans and mice. In each case, the interference by siRNAs was superior to the inhibition of gene expression mediated by single-stranded antisense oligonucleotides. The siRNAs seem to avoid the well documented nonspecific effects triggered by longer double-stranded RNAs in mammalian cells. These observations may open a path toward the use of siRNAs as a reverse genetic and therapeutic tool in mammalian cells.

Mechanisms that silence unwanted gene expression are critical for normal cellular function. Characterized gene silencing mechanisms include a variety of transcriptional and posttranscriptional surveillance processes (1–3). Double-stranded RNA (dsRNA) has been shown to trigger one of these posttranscriptional surveillance processes, in which gene silencing involves the degradation of single-stranded RNA (ssRNA) targets complementary to the dsRNA trigger (4). RNA interference (RNAi) effects triggered by dsRNA have been demonstrated in a number of organisms including plants, protozoa, nematodes, and insects (5). RNAi may play a role in the silencing of mobile elements in *Caenorhabditis elegans* and *Drosophila* (6–9). Similar posttranscriptional gene silencing (PTGS) effects have been implicated as an anti-viral response in plants. PTGS/RNAi seems to be a multistep pathway requiring the processing of the trigger, a facilitated interaction with, and degradation of, the target mRNA. In some cases, these processes may also involve physical amplification of the trigger RNA and long-term maintenance of gene silencing (10, 11).

A key finding from recent work has shown the generation of small (≈21–25 nucleotides) dsRNAs from the input dsRNA during PTGS and RNAi (12–16). These small dsRNAs have been detected in plants, *Drosophila*, and *C. elegans* and have been suggested to serve as guide RNAs for target recognition. In *Drosophila* extracts subjected to RNAi, these small dsRNAs [called short interfering (siRNAs)] resemble breakdown products of an RNase III-like digestion (17). In particular, each strand of the siRNAs carry 5′ phosphate and 3′ hydroxyl termini and 2- or 3-nt 3′ overhangs. siRNAs of 21–22 nucleotides can induce specific degradation when added to *Drosophila* cell extracts (17). Further, a *Drosophila* dsRNA-specific RNase has been identified that can degrade large dsRNA (200 and 500 bp) to small dsRNAs of ≈22 nucleotides. RNAi-triggered inhibition of this ribonuclease significantly reduces the effectiveness of RNAi in *Drosophila* S2 cells (18).

As yet, clear evidence for the generality of an RNAi-like mechanism in vertebrate cells is lacking. Several studies have reported evidence for dsRNA-triggered silencing in particular certain vertebrate systems, early embryos of mice, zebrafish, and *Xenopus*, as well as Chinese hamster ovary cells (19–25). At the same time, numerous reports have described failures to observe gene-specific RNAi effects in different vertebrate systems, demonstrating instead nonspecific effects of dsRNA on gene expression (26–29). These nonspecific effects have not been surprising as there is an extensive literature describing a variety of nonspecific responses induced by dsRNAs in mammalian cells. A major component of the mammalian nonspecific response to dsRNA is mediated by the dsRNA-dependent protein kinase, PKR, which phosphorylates and inactivates the translation factor eIF2α, leading to a generalized suppression of protein synthesis and cell death via both nonapoptotic and apoptotic pathways (30). PKR may be one of several kinases in mammalian cells that can mediate this response (31). A second dsRNA-response pathway involving the dsRNA-induced synthesis of 2′-5′ polyadenylic acid and a consequent activation of a sequence-nonspecific RNase (RNaseL) has also been demonstrated (32). These nonspecific responses to dsRNA, however, do not necessarily preclude the presence of an RNAi-like mechanism in mammalian cells. The activation of PKR by dsRNA has been shown to be length-dependent; dsRNAs of less than 30 nucleotides are unable to activate PKR, and full activation requires ≈80 nucleotides (33, 34). Given the observations that (*i*) 21–25-nt dsRNAs with a characteristic structure can mediate RNAi in cell extracts and that (*ii*) dsRNAs of less than 30 bp do not activate PKR, we set out to determine whether short dsRNAs with an RNase III cleavage structure could trigger a gene-specific RNAi response in model invertebrates and mammalian cells.

Methods

Nucleic Acids. Single-stranded, gene-specific sense and antisense RNA oligomers were synthesized by using 2′-*O*-(tri-isopropyl) silyloxymethyl chemistry by Xeragon AG (Zurich, Switzerland). We have previously shown RNAs produced by this methodology are highly pure and efficiently form RNA duplexes (16, 27). For

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Abbreviations: dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; siRNA, short interfering RNA; RNAi, RNA interference; PTGS, posttranscriptional gene silencing; GFP, green fluorescence protein; CAT, chloramphenicol acetyl transferase; PKR, dsRNA-dependent protein kinase; neo, neomycin phosphotransferase; MEF, mouse embryonic fibroblast; FACS, fluorescence-activated cell sorter.

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studies conducted in *C. elegans*, RNA oligomers were annealed and injected into adults at a concentration of 5 mg/ml as described (16). For experiments conducted using mammalian cells, dsRNA molecules were generated by mixing sense and antisense ssRNA oligomers (100 μg each) in 10 mM Tris·Cl (pH 7.0), 20 mM NaCl (total volume 300 μl), heating to 95°C, and cooling slowly (18 h) to room temperature. The dsRNAs were ethanol-precipitated and resuspended in water at ≈0.5 mg/ml. The integrity and the dsRNA character of the annealed RNAs were confirmed by gel electrophoresis. The sequences of the RNA oligonucleotides used are shown in Table 2, which is published as supplemental data on the PNAS web site, www.pnas.org; the *cat* 22 and 23 ssRNA oligomers were HPLC-purified. Plasmid pEGFP-N3 (CLONTECH) expresses a mammalian-enhanced version of green fluorescent protein (GFP) and neomycin phosphotransferase (neo). Plasmid pcDNA3.CAT (Invitrogen) expresses chloramphenicol acetyl transferase (CAT) and neo.

Cell Culture and Nucleic Acid Transfections. All mammalian cells were grown in DMEM (Life Technologies, Rockville, MD) supplemented with 10% (vol/vol) FBS (Gemini Biological Products, Calabasas, CA). Primary mouse embryonic fibroblasts (MEFs) from wild-type 1129 mouse embryos (a gift of J. Bell, Univ. of Ottawa, Ontario, Canada; ref. 31) were expanded to generate a more homogenous cell line and were used at passages 20–50 (35). 293 is a human embryonic kidney cell line (36); HeLa is a human epithelial cell line derived from a cervical adenocarcinoma [American Type Culture Collection (ATTC) no. CCL-2]. Plasmid/RNA cotransfection of mammalian cells was mediated by using the cationic lipid Lipofectamine (GIBCO) and the propriety plus reagent (Life Technologies). Cells were seeded ≈18 h before transfection and were transfected at ≈70–80% confluency. Plasmid DNA was complexed with the plus reagent (4–6 μl/2 μg DNA) in DMEM for ≈15 min. RNAs were added 5–10 min into the plasmid/plus reagent incubation. Lipofectamine diluted in DMEM was added to the plasmid/plus reagent/RNA mixture, and complexation was continued for an additional 15 min. The amount of Lipofectamine added (8–15 μg) was based on the total weight of nucleic acid (DNA and RNA) used and a weight to weight ratio of nucleic acid to lipid of 1:4. The amount of RNA used was adjusted to account for the variations in the sizes of RNA. For small RNAs (21–27 nucleotides), 70 pmols of ssRNA and dsRNA was used, corresponding to ≈0.5 μg of a 22-nt ssRNA and 1 μg of 22-nt dsRNA. For the larger RNAs (78–81 nucleotides), ≈30 pmols of RNA was used (0.85 μg of ssRNA and 1.7 μg of dsRNA). Three hours after initiation of transfection, DMEM supplemented with 20% (vol/vol) FBS was added to the cells.

Analysis of Gene Expression. The *C. elegans unc-22* gene encodes an abundant striated muscle component that results in a characteristic twitching phenotype. Animals were scored for the twitching phenotype as described (16). GFP expression was assessed in mammalian cells by fluorescence-activated cell sorter (FACS; FacsCaliber, Becton Dickinson) by using pcDNA3.CAT-transfected cells to control for background fluorescence. CAT expression was assessed by using an ELISA-based assay (Roche Molecular Biochemicals). Total protein was determined by using the Bradford method as described (27). Poly(A)$^+$ RNA was purified from MEFs by using GTC extraction, oligo(dT) cellulose chromatography, and DNase digestion to remove residual plasmid DNA. After electrophoresis [1.2% agarose/1 × 4-morpholinepropanesulfonic acid (Mops)/5.0% formaldehyde] and Northern blot transfer, filters were sequentially hybridized with random prime-labeled cDNA probes corresponding to *egfp* and *neo*. Hybridization intensities were measured by using a BAS150 PhosphorImager (Molecular Dynamics), and pixel densities were calculated by using IMAGE READER 1.4 and IMAGE GAUGE 3.0 (Fuji).

Table 1. Short RNase III-like products can induce specific interference in *C. elegans*

Injection	Fraction affected (number scored)
unc-22 siRNA 23 nts	1.4% (145)
unc-22 siRNA 24 nts	3.6% (279)
unc-22 siRNA 25 nts	16.3% (768)
unc-22 sense ssRNA 25 nts	0% (>1100)
unc-22 antisense ssRNA 25 nts	0% (>600)
unc-22 dsRNA 81 nts	88.9% (180)
egfp siRNA 22 nts	0% (>300)
egfp siRNA 23 nts	0% (>300)
egfp siRNA 24 nts	0% (>300)
egfp siRNA 25 nts	0% (>300)
No injection	0% (>300)

dsRNA molecules were formed with each strand carrying a 5′-PO_4, 3′-OH, and 2-base 3′ overhangs. These were injected into adult *C. elegans* as described in *Methods*. Percentages shown denote portion of progeny broods that show a specific decrease in *unc-22* function as evidenced by twitching behavior in 330 μM levamisole. Numbers in parenthesis are total numbers of animals scored. nts, nucleotides.

Cell Survival and *in Vitro* Kinase Assays. To assay cell survival, MEFs were plated in 96-well plates ≈18 h before transfection and were transfected at ≈70–80% confluency by using Lipofactamine as a carrier. RNA transfections were conducted as above, except for the omission of the plus complexation step, and using 1/10th the amount of RNA and lipid and 1/10th the volume of medium. Cell viability was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) labeling reagent as described by the manufacturer (Roche Molecular Biochemicals) 48 h after initiation of transfection. *In vitro* kinase reactions were conducted in a final volume of 12.5 ml by using 100 mM [γ-^{32}P]ATP (specific activity 1 Ci/mM, Amersham Pharmacia), 100 mM ATP (Sigma) in 20 mM Hepes (pH 7.5), 90 mM KCl, 5 mM MgOAc, 1 mM DTT, and an equal amount of cell lysate prepared from 1 × 10^6 human Jurkat T lymphocytes treated with 100 units/ml of rhIFN-β for 24 h before lysis (lysis buffer: 20 mM Hepes/120 mM KCl/5 mM MgOAc/1 mM benzamidine/1 mM DTT/1% Nonidet P-40). dsRNA (1 μg/ml) was added to each reaction mixture, and the reactions were incubated for 10 min at 30°C. Reactions were quenched by addition of an equal volume of 2 times sample buffer (2 times sample buffer: 62.5 mM Tris·Cl, pH 6.8/10% glycerol/2% SDS/0.0125% bromophenol blue/5% β-mercaptoethanol), boiled for 2 min, and subjected to electrophoresis [10% (vol/vol) SDS/PAGE]. Labeled proteins were visualized by autoradiography of dried gels.

Results

Short RNase III-Like Products Can Induce Inhibition of Gene Expression in Invertebrate Cells. A series of dsRNAs with characteristics of siRNAs (5′ phosphate, 3′ hydroxyl, and 2 base 3′ overhangs on each strand) were generated from chemically synthesized ssRNAs. The siRNAs varied from 21–27 nucleotides and had sequences that matched three different target RNAs, *unc-22*, *cat*, and *egfp* (for sequences see Table 2).

To determine whether siRNAs can be used directly to inhibit gene expression we first assessed interference in *C. elegans* by using siRNAs corresponding to *C. elegans unc-22* (Table 1). *unc-22* provides a sensitive and specific assay for genetic interference as this is the only gene in the *C. elegans* genome that can mutate by loss of function to give a twitching phenotype. *unc-22*

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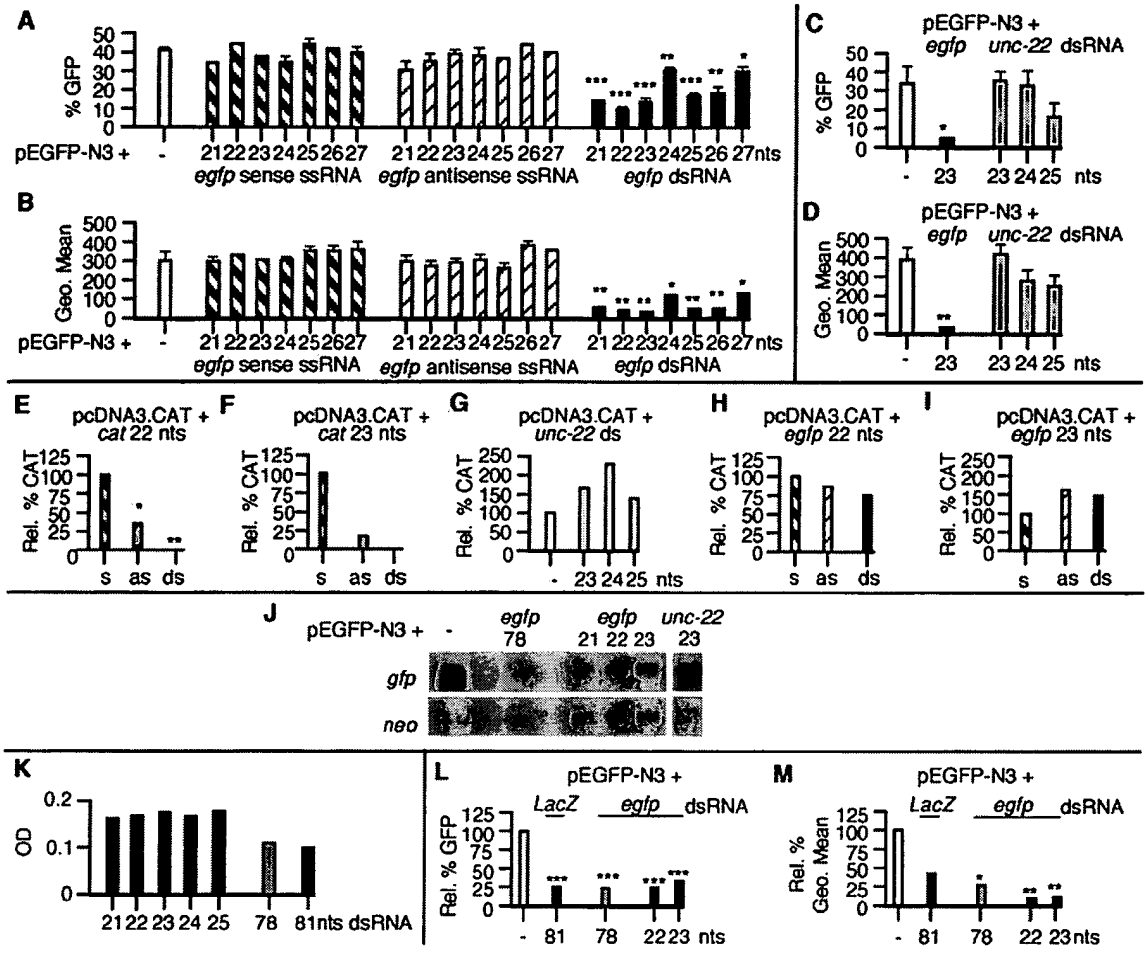

Fig. 1. Gene-specific inhibition of expression in MEFs by siRNAs. MEFs transfected with plasmid DNA, ssRNAs, and dsRNAs were harvested 48 h after transfection and were assayed for (*A–D*) GFP expression by FACS analysis (each transfection was assayed in triplicate and data are shown as mean ± SEM). *A* and *C* show the percentage of GFP-positive cells and *B* and *D* show the fluorescence intensity (Geo Mean) of GFP-positive cells. (*E–I*) CAT expression (each transfection condition was assayed in triplicate; data in *E* are normalized to the amount of CAT pg/μg of protein observed in pcDNA3.CAT-transfected cells; data in *F–I* are normalized to the amount of CAT pg/μg of protein in plasmid and sense ssRNA-transfected cells. s, sense ssRNA; as, antisense ssRNA). (*J*) *egfp* and *neo* RNA levels by Northern analysis of poly(A)$^+$ mRNA. (*K*) Cell survival (assayed in duplicate and shown as a mean $OD_{560-650}$; dsRNAs of 21–25 and 78 nucleotides correspond to *egfp*; the dsRNA of 81 nucleotides corresponds to *LacZ*). (*L* and *M*) GFP expression by FACS analysis (data are shown as relative percentage normalized to pEGFP-N3-transfected cells). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

siRNAs induced a decrease in *unc-22* gene expression as measured by the presence of the twitching phenotype in the progeny of injected adults. Small dsRNAs of 23, 24, and 25 nucleotides produced interference with the 25-nt *unc-22* siRNA inducing the highest fraction of animals with an affected phenotype (16.3%). As a control, siRNAs directed against an unrelated sequence (*egfp*) induced no phenotypic changes (Table 1).

21–23-nt dsRNAs Inhibit Expression in MEFs. To test whether small dsRNA molecules can specifically inhibit gene expression in vertebrate cells, we cotransfected MEFs with expression plasmids encoding GFP (pEGFP-N3) and CAT (pcDNA3.CAT), and synthetic siRNAs corresponding to *egfp*, *cat*, or *unc-22* (Fig. 1). The *egfp* dsRNAs (21–27 nucleotides) all inhibited GFP expression in MEFs. The 22- and 23-nt *egfp* siRNAs (20 and 21 nucleotides base-paired with 2-nt 3′ overhangs) showed the greatest degree of inhibition, both with respect to the total number of cells expressing GFP (Fig. 1*A*) and the fluorescence intensity of the GFP expression observed in GFP-positive cells (Fig. 1*B*). In contrast, *unc-22* dsRNAs of 23–25 nucleotides had no significant effect on GFP expression (Fig. 1 *C* and *D*).

To further assess the efficacy and specificity of the inhibition mediated by siRNAs in mammalian cells, we used a second reporter, CAT (Fig. 1 *E–I*). *cat* siRNAs of 22 and 23 nucleotides completely inhibited CAT expression (Fig. 1 *E* and *F*), whereas *unc-22* and *egfp* dsRNAs had no little or no effect on CAT expression (Fig. 1 *G–I*). Although no antisense effect had been seen by using GFP as a reporter, the *cat* ssRNA antisense oligomers partially inhibited CAT expression. However, the siRNA-mediated inhibition was more potent (≈1.5-fold), suggesting that the gene silencing mediated by the small dsRNAs can be distinguished from a purely antisense-based mechanism.

To analyze this inhibition of *egfp* expression at an RNA level, poly(A)$^+$ RNA was purified from transfected MEFs and subjected to Northern analysis by using cDNA probes corresponding to *egfp* and *neo*, both encoded by the pEGFP-N3 plasmid (Fig. 1*J*). Quantitative PhosphorImager analysis showed a decrease in the levels of the *egfp* mRNA obtained from cells cotransfected

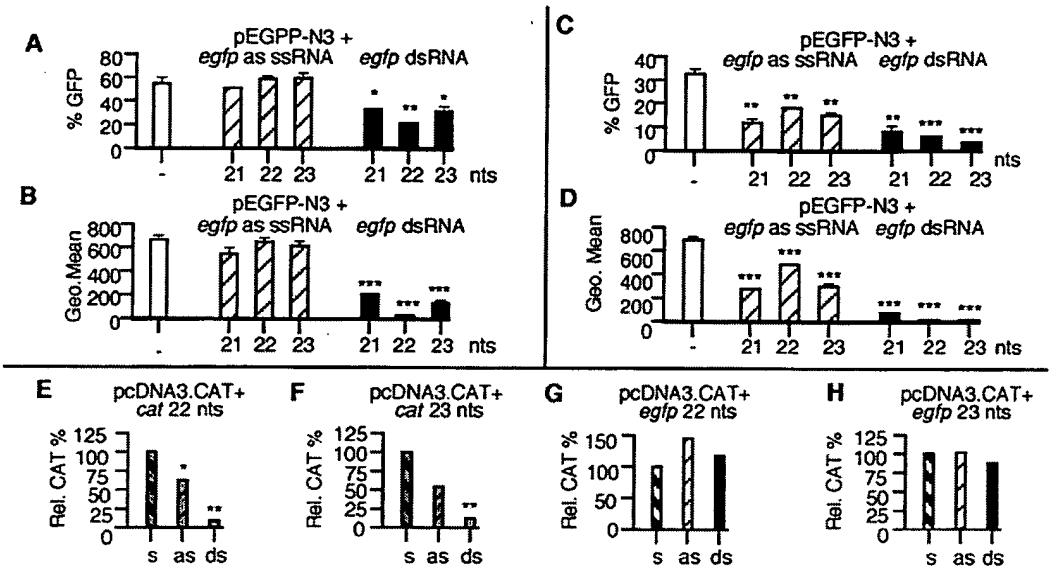

Fig. 2. siRNA-mediated gene silencing in human cells. (*A* and *B*) 293 and (*C* and *D*) HeLa cells transfected with pEGFP-N3 and antisense (as) ssRNAs and dsRNAs were harvested 48 h after transfection and were assayed for GFP expression by FACS analysis (assayed in triplicate; data are shown as mean ± SEM). *A* and *C* show the percentage of GFP-positive cells and *B* and *D* show the fluorescence intensity (Geo Mean) of GFP-positive cells. (*E–H*) HeLa cells transfected with pcDNA3-CAT, ssRNAs, and dsRNAs were harvested 48 h after transfection and assayed for CAT expression (assayed in triplicate and normalized to the amount of CAT pg/μg of protein observed in plasmid plus sense-transfected cells. s, sense ssRNA; as, antisense ssRNA). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

with the pEGFP-N3 plasmid and the 21-, 22-, and 23-nt *egfp* siRNAs, compared with cells transfected only with the GFP plasmid. The percentage decrease was ≈60% for all three *egfp* siRNAs when compared with the levels of *egfp* mRNA in cells transfected only with plasmid. Importantly, no effect was seen on the levels of the *neo* transcript compared with plasmid-only transfected cells, indicating that the inhibition induced by the small *egfp* dsRNAs was sequence-specific. Consistent with this hypothesis, the 23-nt dsRNA corresponding to the *C. elegans unc-22* gene had no effect on either *egfp* or *neo* expression.

To follow the fate of cells transfected with siRNAs and larger dsRNAs, we assayed MEF cell survival (Fig. 1*K*). Longer dsRNAs (78 or 81 nucleotides with flush ends) induced a substantial degree of cell death (up to 50%) in a 48-h period, whereas the smaller dsRNAs had a minimal effect on the growth of cells. By examining the effect of the larger dsRNAs on gene expression, we observed that the larger dsRNAs (78 or 81 nucleotides) induced a sequence nonspecific decrease of 75% in the percentage of cells expressing GFP (Fig. 1*L*) and in CAT protein levels (data not shown), compared with plasmid controls. This nonspecific decrease in gene expression is consistent with previous data from numerous mammalian systems and contrasts with the specific gene silencing the 78-nt *egfp* dsRNA induces in *Drosophila* S2 cells (27). However, it should be noted that the decrease in transgene expression after siRNA transfection could be distinguished from the nonspecific inhibition by examination of the GFP fluorescence intensity seen in viable cells. The fluorescence intensity of GFP expression best illustrates a change in the total amount of GFP made by a live cell and therefore is less influenced by nonspecific cell death. Although some decrease (≈60%) in the fluorescence intensity was seen by using the larger ≈80-nt dsRNA molecules (irrespective of sequence), the *egfp* siRNAs of 22 and 23 nucleotides consistently reduced the intensity of the GFP signal (by ≈90%) to near background levels (Fig. 1*M*). The difference in specificity between the longer dsRNAs and siRNAs could also be seen at an RNA level were the 78-nt *egfp* dsRNA induced a significant decrease in both the *egfp* and *neo* transcripts, whereas the siRNAs inhibited only *egfp* (Fig. 1*J*).

Inhibition of Gene Expression in Human Somatic Cells. To date, there has been no evidence of an RNAi-like process occurring in human somatic cells. To determine whether siRNAs could also specifically inhibit gene expression in human cells, we cotransfected two commonly used human cell lines, the embryonic kidney cell line 293 and the epithelial carcinoma cell line HeLa, with plasmids and RNA (Fig. 2). All of the *egfp* siRNAs tested inhibited GFP gene expression in 293 (Fig. 2 *A* and *B*) and HeLa (Fig. 2 *C* and *D*) cells, with the 22- and 23-nt *egfp* siRNAs inducing the greatest decrease in GFP expression. In 293 cells cotransfected with pEGFP-N3 and the 22-nt *egfp* siRNA, the intensity of GFP expression was reduced to near background levels (Fig. 2*B*). Similar results were seen in HeLa cells cotransfected with pEGFP-N3 and the 22- or 23-nt *egfp* siRNAs (Fig. 2*D*). dsRNAs corresponding to *unc-22* had no effect on GFP expression in these cells (data not shown). The siRNA-triggered inhibition of GFP expression was dose-dependent in that doubling the amount of dsRNA (from 70 to 140 pmols) decreased GFP intensity by an additional 25% for the *egfp* 22-nt siRNA and by 45% for the *egfp* 23-nt siRNA. CAT expression was also significantly inhibited by siRNAs corresponding to *cat* (Fig. 2 *E* and *F*) in HeLa cells. Again, the inhibition mediated by the siRNAs was significantly higher than that seen by using ssRNA antisense oligomers. Cotransfection of the pcDNA3.CAT plasmid and the *egfp* siRNAs of the same size and of similar GC/AT complexity had no effect on CAT expression (Fig. 2 *G* and *H*).

siRNA-Mediated Inhibition of Gene Expression Is Independent of Nonspecific Interference Pathways Activated by Larger dsRNAs. It has been reported that small blunt-ended dsRNAs of less than 30 bp do not activate PKR (34). Indeed, at high concentrations these short dsRNAs can competitively inhibit activation of PKR by larger dsRNAs. Similarly, the synthetic siRNAs used in this study did not activate PKR (Fig. 3*A*) and inhibited the activation of

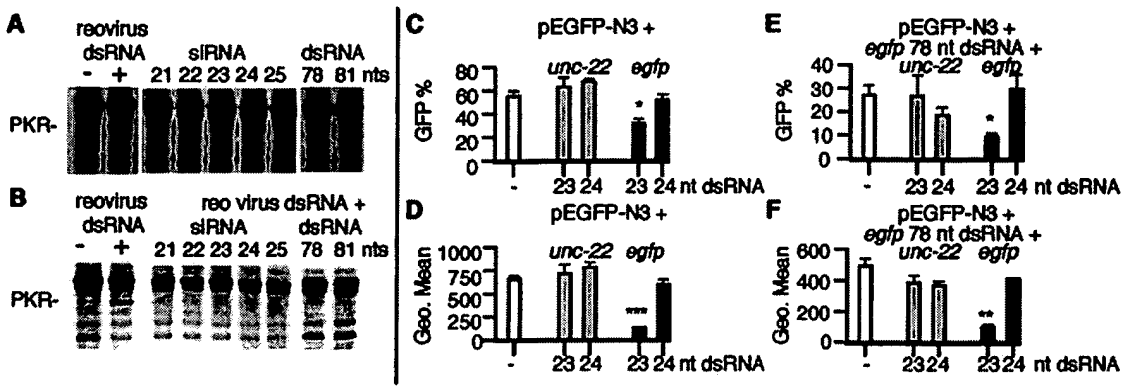

Fig. 3. siRNAs and mammalian dsRNA-dependent pathways. To detect PKR autophosphorylation, we performed *in vitro* kinase assays as described in *Methods*. (*A*) *In vitro* kinase reactions were performed without exogenous RNA (−) or with 1 μg/ml of reovirus dsRNA or 1 μg/ml of siRNA (21–25 nucleotides), or 1 μg/ml of 78- or 81-nt dsRNA. (*B*) *In vitro* kinase competition assays were performed by using si- and dsRNAs. Reactions were performed without exogenous RNA (−) or 1 μg/ml of reovirus RNA, or 75-fold excess siRNA (21–25 nucleotides) or 78- or 81-nt dsRNA, plus reovirus dsRNA (1 μg/ml). siRNAs of 21–25 nucleotides and dsRNA of 78 nucleotides corresponded to *egfp* (the 81-nt dsRNA corresponds to *LacZ*). (*C* and *D*) 293 cells transfected with pEGFP-N3 and *unc-22* or *egfp* siRNAs, and (*E* and *F*) 293 cells transfected with pEGFP-N3 and 78 *egfp* dsRNA and *unc-22* or *egfp* siRNAs were assayed for GFP expression by FACS analysis 48 h after transfection (each transfection was assayed in triplicate; data are shown as mean ± SEM). *B* and *D* show the percentage of GFP-positive cells and *C* and *E* show the fluorescence intensity (Geo Mean) of GFP-positive cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

PKR by a large viral dsRNA (Fig. 3*B*). Interestingly, in this assay we were unable to detect activation of PKR by the 78- and 81-nt dsRNAs, despite observing a substantial level of cell death, suggesting that other dsRNA-dependent kinases or other pathways may be contributing in MEFs to the decrease in gene expression and cell death observed with these RNAs.

To see whether the small dsRNAs could block the toxic effect of the larger dsRNAs in cells, we cotransfected 293 cells with the pEGFP-N3 plasmid, the *egfp* 78-nt dsRNA, and the *unc-22* 23- or 24-nt siRNAs or the *egfp* 23- or 24-nt siRNAs (Fig. 3 *C–F*). The cell death induced by the 78-nt *egfp* dsRNA was not inhibited by the *unc-22* or *egfp* siRNAs (Fig. 3 *C* and *D* vs. *E* and *F*) but importantly the 78-nt *egfp* dsRNA did not block the specific inhibition of GFP expression mediated by the 23-nt *egfp* siRNA. This result suggests that the siRNA-mediated gene silencing mechanism is independent of nonspecific responses of mammalian cells to dsRNA.

Discussion

A consistent observation of PTGS and RNAi in several species has been the detection of small dsRNAs (≈21–25 nucleotides) and siRNAs derived from the triggering dsRNA. These small dsRNAs have been observed irrespective of whether the initiating dsRNA is delivered directly, is derived from a viral RNA, or is produced from a transgene (12–17). These findings and further biochemical analysis (18) have suggested that the generation of siRNAs represents a critical step in the RNAi/PTGS mechanism. We now present evidence that these siRNAs can have direct effects on gene expression in *C. elegans* and mammalian cell culture *in vivo*. Our results in mammalian cells are particularly striking in that previous attempts to assay RNAi effects in vertebrate somatic cells have encountered effects that were predominately gene-nonspecific (26–29). We propose that the small size of the siRNAs avoids the induction of the nonspecific responses of mammalian cells to dsRNA.

Several models have been put forward to explain RNAi, in particular the mechanisms by which siRNAs interact with the target mRNA and thus facilitate its degradation (12–15, 17, 37). It has been proposed that the siRNAs act as a guide for the enzymatic complex required for the sequence-specific cleavage of the target mRNA. Evidence for the role of siRNA as a guide includes cleavage of the target mRNA at regular intervals of ≈21–23 nucleotides in the region corresponding to the input dsRNA (13), with the exact cleavage sites corresponding to the middles of sequences covered by individual 21- or 22-nt siRNAs (17). Although mammals and lower organisms seem to share dsRNA-triggered responses that involve a related intermediate (siRNAs), it is likely that there will be differences as well as similarities in the underlying mechanism.

Several of the proteins shown to play key roles in RNA-triggered gene silencing in plants and invertebrates share homology with potential coding regions from the human or other vertebrate genomes. These include putative RNA-dependent polymerases (RdRp; refs. 38–41), the RDE-1/Argonaute family (8), and a variety of putative helicases and nucleases (9, 18, 42–44). Mammalian homologs of the RNAi-associated *Drosophila* RNase III have been identified (45, 46). Importantly, one of these putative RNases has been shown to generate small dsRNA molecules of ≈22 nucleotides from larger dsRNAs (18). However, even in invertebrate systems, the precise role of these factors in RNAi remains to be elucidated. Because factors from each of these homology classes have identified roles in normal physiology and development (i.e., beyond genome surveillance), a full analysis of the reaction mechanisms in the different biological systems may be needed before a clear picture of the commonality between RNAi in these different systems will emerge.

Our experiments do not address possible differences in mechanism between invertebrate and vertebrate systems, although we observed some variation between the different assay systems in the optimal size and effectiveness of the inhibiting dsRNA. These differences could be gene-, species-, cell type-, or assay-specific; it will be particularly interesting to determine whether there are species-dependent differences in the length or structure of natural siRNAs. It is not yet clear what roles RNAi/PTGS might play in mammalian systems. RNAi-related silencing mechanisms in plant and invertebrate systems have been implicated in the silencing of viruses and transposons. Mammalian genomes have a need to cope with a considerable load of viruses, selfish DNA, and aberrant transcription. RNAi-related mechanisms could certainly function as a part of the defense network for any or all of these genomic hazards. Alternatively, specific gene silencing by dsRNA could function in normal mammalian gene regulation, e.g., in imprinting or X inactivation (47).

Because of the efficacy and ease with which RNAi can be induced, RNAi has been rapidly exploited in *C. elegans* and *Drosophila* as a reverse genetics tool (48). Currently, the principal method used to reduce gene expression in mammalian cells utilizes antisense sequences in the form of single-stranded oligonucleotides and transcripts. The interaction of antisense sequences with mRNA through Watson–Crick base-pairing leads to a decrease in gene expression by several possible mechanisms, including the activation of RNaseH, which cleaves RNA/DNA duplexes, and the inhibition of RNA processing and/or translational blockade (49). Several issues have limited wider use of antisense technology. Problems have included a lack of suitable target sequences within a given mRNA caused by RNA secondary folding, which necessitates screening of multiple antisense sequences to identify those that mediate the greatest level of inhibition and inefficient delivery *in vitro* and *in vivo*. We have tested only a limited number of siRNAs in mammalian cells but as yet all of the siRNAs that were tested produced specific inhibition of gene expression, and the siRNAs seem to be very stable and thus may not require the extensive chemical modifications that ssRNA antisense oligonucleotides require to enhance the *in vivo* half-life. Our initial experiments suggest that siRNAs may be useful for triggering RNAi-like responses that could be used as functional genomics and therapeutic tools. Certain applications may be facilitated by the simple transfection protocols that we have used, whereas other applications may benefit from further optimization and additional exploration of the RNAi mechanism.

Note. A recent report by Elbashir *et al.* (50) describes a specific interference response in mammalian cells by using 21-nt siRNAs.

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1. Wolffe, A. P. & Matzke, M. A. (1999) *Science* **286,** 481–486.
2. Frischmeyer, P. A. & Dietz, H. C. (1999) *Hum. Mol. Genet.* **8,** 1893–1900.
3. Mitchell, P. & Tollervey, D. (2000) *Curr. Opin. Genet. Dev.* **10,** 193–198.
4. Fire, A. (1999) *Trends Genet.* **15,** 358–363.
5. Cogoni, C. & Macino, G. (2000) *Curr. Opin. Genet. Dev.* **10,** 638–643.
6. Kasschau, K. D. & Carrington, J. C. (1998) *Cell* **95,** 461–470.
7. Llave, C., Kasschau, K. D. & Carrington, J. C. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 13401–13406. (First Published November 14, 2000; 10.1073/pnas.230334397)
8. Tabara, H., Sarkissian, M., Kelly, W. G., Fleenor, J., Grishok, A., Timmons, L., Fire, A. & Mello, C. C. (1999) *Cell* **99,** 123–132.
9. Ketting, R. F., Haverkamp, T. H., van Luenen, H. G. & Plasterk, R. H. (1999) *Cell* **99,** 133–141.
10. Matzke, M. A., Matzke, A. J., Pruss, G. J. & Vance, V. B. (2001) *Curr. Opin. Genet. Dev.* **11,** 221–227.
11. Carthew, R. W. (2001) *Curr. Opin. Cell Biol.* **13,** 244–248.
12. Hamilton, A. J. & Baulcombe, D. C. (1999) *Science* **286,** 950–952.
13. Zamore, P. D., Tuschl, T., Sharp, P. A. & Bartel, D. P. (2000) *Cell* **101,** 25–33.
14. Hammond, S. M., Bernstein, E., Beach, D. & Hannon, G. J. (2000) *Nature (London)* **404,** 293–296.
15. Yang, D., Lu, H. & Erickson, J. W. (2000) *Curr. Biol.* **10,** 1191–1200.
16. Parrish, S., Fleenor, J., Xu, S., Mello, C. & Fire, A. (2000) *Mol. Cell.* **6,** 1077–1087.
17. Elbashir, S. M., Lendeckel, W. & Tuschl, T. (2001) *Genes Dev.* **15,** 188–200.
18. Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. (2001) *Nature (London)* **409,** 363–366.
19. Wianny, F. & Zernicka-Goetz, M. (2000) *Nat. Cell Biol.* **2,** 70–75.
20. Svoboda, P., Stein, P., Hayashi, H. & Schultz, R. M. (2000) *Development (Cambridge, U.K.)* **127,** 4147–4156.
21. Wargelius, A., Ellingsen, S. & Fjose, A. (1999) *Biochem. Biophys. Res. Commun.* **263,** 156–161.
22. Li, Y. X., Farrell, M. J., Liu, R., Mohanty, N. & Kirby, M. L. (2000) *Dev. Biol.* **217,** 394–405.
23. Nakano, H., Amemiya, S., Shiokawa, K. & Taira, M. (2000) *Biochem. Biophys. Res. Commun.* **274,** 434–439.
24. Ui-Tei, K., Zenno, S., Miyata, Y. & Saigo, K. (2000) *FEBS Lett.* **479,** 79–82.
25. Oelgeschlager, M., Larrain, J., Geissert, D. & De Robertis, E. M. (2000) *Nature (London)* **405,** 757–763.
26. Tuschl, T., Zamore, P. D., Lehmann, R., Bartel, D. P. & Sharp, P. A. (1999) *Genes Dev.* **13,** 3191–3197.
27. Caplen, N. J., Fleenor, J., Fire, A. & Morgan, R. A. (2000) *Gene* **252,** 95–105.
28. Oates, A. C., Bruce, A. E. & Ho, R. K. (2000) *Dev. Biol.* **224,** 20–28.
29. Zhao, Z., Cao, Y., Li, M. & Meng, A. (2001) *Dev. Biol.* **229,** 215–223.
30. Clemens, M. J. & Elia, A. (1997) *J. Interferon Cytokine Res.* **17,** 503–524.
31. Abraham, N., Stojdl, D. F., Duncan, P. I., Methot, N., Ishii, T., Dube, M., Vanderhyden, B. C., Atkins, H. L., Gray, D. A., McBurney, M. W., *et al.* (1999) *J. Biol. Chem.* **274,** 5953–5962.
32. Player, M. R. & Torrence, P. F. (1998) *Pharmacol. Ther.* **78,** 55–113.
33. Minks, M. A., West, D. K., Benvin, S. & Baglioni, C. (1979) *J. Biol. Chem.* **254,** 10180–10183.
34. Manche, L., Green, S. R., Schmedt, C. & Mathews, M. B. (1992) *Mol. Cell. Biol.* **12,** 5238–5248.
35. Todaro, G. J. & Green, H. (1963) *J. Cell Biol.* **17,** 299–313.
36. Graham, F. L., Smiley, J., Russell, W. L. & Nairn, R. (1977) *J. Gen. Virol.* **36,** 59–72.
37. Bass, B. L. (2000) *Cell* **101,** 235–238.
38. Cogoni, C. & Macino, G. (1999) *Nature (London)* **399,** 166–169.
39. Smardon, A., Spoerke, J. M., Stacey, S. C., Klein, M. E., Mackin, N. & Maine, E. M. (2000) *Curr. Biol.* **10,** 169–178.
40. Dalmay, T., Hamilton, A., Rudd, S., Angell, S. & Baulcombe, D. C. (2000) *Cell* **101,** 543–553.
41. Mourrain, P., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J. B., Jouette, D., Lacombe, A. M., Nikic, S., Picault, N., *et al.* (2000) *Cell* **101,** 533–542.
42. Wu-Scharf, D., Jeong, B., Zhang, C. & Cerutti, H. (2000) *Science* **290,** 1159–1162.
43. Dernburg, A. F., Zalevsky, J., Colaiacovo, M. P. & Villeneuve, A. M. (2000) *Genes Dev.* **14,** 1578–1583.
44. Domeier, M. E., Morse, D. P., Knight, S. W., Portereiko, M., Bass, B. L. & Mango, S. E. (2000) *Science* **289,** 1928–1931.
45. Matsuda, S., Ichigotani, Y., Okuda, T., Irimura, T., Nakatsugawa, S. & Hamaguchi, M. (2000) *Biochim. Biophys. Acta* **1490,** 163–169.
46. Wu, H., Xu, H., Miraglia, L. J. & Crooke, S. T. (2000) *J. Biol. Chem.* **275,** 36957–36965.
47. Sharp, P. A. (2001) *Genes Dev.* **15,** 485–490.
48. Kuwabara, P. E. & Coulson, A. (2000) *Parasitol. Today* **16,** 347–349.
49. Crooke, S. T. (1999) *Biochim. Biophys. Acta* **1489,** 31–44.
50. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. & Tuschl, T. (2001) *Nature (London)* **411,** 494–498.

ATTACHMENT E

ogy), respectively. Staining specificity was controlled by single staining, as well as by using secondary antibodies in the absence of the primary stain.

Generation of target cells

Target cells displaying a membrane-integral version of either wild-type HEL or a mutant[10] exhibiting reduced affinity for HyHEL10 ([R^{21}, D^{101}, G^{102}, N^{103}] designated HEL*) were generated by transfecting mouse J558L plasmacytoma cells with constructs analogous to those used[10] for expression of soluble HEL/HEL*, except that 14 Ser/Gly codons, the $H2K^b$ transmembrane region, and a 23-codon cytoplasmic domain were inserted immediately upstream of the termination codon by polymerase chain reaction. For mHEL–GFP, we included the EGFP coding domain in the Ser/Gly linker. Abundance of surface HEL was monitored by flow cytometry and radiolabelled-antibody binding using HyHEL5 and D1.3 HEL-specific monoclonal antibodies, for which the mutant HELs used in this work show unaltered affinities[10].

Interaction assays

For B-cell/target interaction assays, splenic B cells from 3-83 or MD4 transgenic mice[28,29] carrying (IgM + IgD) BCRs specific for HEL or $H2K^k$/$H2K^b$were freshly purified on Lympholyte and incubated with a twofold excess of target cells in RPMI, 50 mM HEPES pH 7.4, for the appropriate time at 37 °C before being applied to polylysine-coated slides. Cells were fixed in 4% paraformaldehyde/PBS or methanol and permeabilized with PBS/0.1% Triton X-100 before immunofluorescence. We acquired confocal images using a Nikon E800 microscope attached to BioRad Radiance Plus scanning system equipped with 488-nm and 543-nm lasers, as well as differential interference contrast for transmitted light. GFP fluorescence in living cells in real time was visualized using a Radiance 2000 and Nikon E300 inverted microscope. Images were processed using BioRad Lasersharp 1024 or 2000 software to provide single plane images, confocal projections or slicing.

Antigen presentation

Presentation of HEL epitopes to T-cell hybridomas 2G7 (specific for I-E^k[HEL^{1-18}]) and 1E5 (specific for I-E^d[$HEL^{108-116}$]) by transfectants of the LK35.2 B-cell hybridoma expressing an HEL-specific IgM BCR was monitored as described[10].

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1. Lanzavecchia, A. Antigen-specific interaction between T and B cells. *Nature* **314**, 537–539 (1985).
2. Klaus, G. G., Humphrey, J. H., Kunkl, A. & Dongworth, D. W. The follicular dendritic cell: its role in antigen presentation in the generation of immunological memory. *Immunol. Rev.* **53**, 3–28 (1980).
3. Tew, J. G., Kosco, M. H., Burton, G. F. & Szakal, A. K. Follicular dendritic cells as accessory cells. *Immunol. Rev.* **117**, 185–211 (1990).
4. Kosco-Vilbois, M. H., Gray, D., Scheidegger, D. & Julius, M. Follicular dendritic cells help resting B cells to become effective antigen-presenting cells: induction of B7/BB1 and upregulation of major histocompatibility complex class II molecules. *J. Exp. Med.* **178**, 2055–2066 (1993).
5. Schamel, W. W. & Reth, M. Monomeric and oligomeric complexes of the B cell antigen receptor. *Immunity* **13**, 5–14 (2000).
6. Taylor, R. B., Duffus, W. P. H., Raff, M. C. & de Petris, S. Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by anti-immunoglobulin antibody. *Nature* **233**, 225–227 (1971).
7. Schreiner, G. F. & Unanue, E. R. Capping and the lymphocyte: models for membrane reorganisation. *J. Immunol.* **119**, 1549–1551 (1977).
8. Cheng, P. C., Dykstra, M. L., Mitchell, R. N. & Pierce, S. K. A role for lipid rafts in B cell antigen receptor signaling and antigen targeting. *J. Exp. Med.* **190**, 1549–1560 (1999).
9. Weintraub, B. C. *et al.* Entry of B cell receptor into signaling domains is inhibited in tolerant B cells. *J. Exp. Med.* **191**, 1443–1448 (2000).
10. Batista, F. D. & Neuberger, M. S. Affinity dependence of the B cell response to antigen: a threshold, a ceiling, and the importance of off-rate. *Immunity* **8**, 751–759 (1998).
11. Nemazee, D. & Burki, K. Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. *Nature* **337**, 562–566 (1989).
12. Hartley, S. B. *et al.* Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. *Nature* **353**, 765–769 (1991).
13. Dustin, M. L. *et al.* Low affinity interaction of human and rat T cell adhesion molecule CD2 with its ligands aligns adhering membranes to achieve high physiological affinity. *J. Biol. Chem.* **272**, 30889–30898 (1997).
14. Lang, J. *et al.* B cells are exquisitely sensitive to central tolerance and receptor editing by ultralow affinity, membrane-bound antigen. *J. Exp. Med.* **184**, 1685–1697 (1996).
15. Valitutti, S., Muller, S., Cella, M., Padovan, E. & Lanzavecchia, A. Serial triggering of many T-cell receptors by a few peptide–MHC complexes. *Nature* **375**, 148–151 (1995).
16. Monks, C. R., Freiberg, B. A., Kupfer, H., Sciaky, N. & Kupfer, A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* **395**, 82–86 (1998).
17. Wulfing, C. & Davis, M. M. A receptor/cytoskeletal movement triggered by costimulation during T cell activation. *Science* **282**, 2266–2269 (1998).
18. Grakoui, A. *et al.* The immunological synapse: a molecular machine controlling T cell activation. *Science* **285**, 221–227 (1999).
19. Leupin, O., Zaru, R., Laroche, T., Muller, S. & Valitutti, S. Exclusion of CD45 from the T-cell receptor signaling area in antigen-stimulated T lymphocytes. *Curr. Biol.* **10**, 277–280 (2000).
20. Cagan, R. L., Kramer, H., Hart, A. C. & Zipursky, S. L. The bride of sevenless and sevenless interaction: internalization of a transmembrane ligand. *Cell* **69**, 393–399 (1992).
21. Huang, J. F. *et al.* TCR-mediated internalization of peptide–MHC complexes acquired by T cells. *Science* **286**, 952–954 (1999).
22. Hwang, I. *et al.* T cells can use either T cell receptor or CD28 receptors to absorb and internalize cell surface molecules derived from antigen-presenting cells. *J. Exp. Med.* **191**, 1137–1148 (2000).
23. Batista, F. D. & Neuberger, M. S. B cells extract and present immobilized antigen: implications for affinity discrimination. *EMBO J.* **19**, 513–520 (2000).
24. Casten, L. A., Lakey, E. K., Jelachich, M. L., Margoliash, E. & Pierce, S. K. Anti-immunoglobulin augments the B-cell antigen-presentation function independently of internalization of receptor-antigen complex. *Proc. Natl Acad. Sci. USA* **82**, 5890–5894 (1985).
25. Siemasko, K., Eisfelder, B. J., Williamson, E., Kabak, S. & Clark, M. R. Signals from the B lymphocyte antigen receptor regulate MHC class II containing late endosomes. *J. Immunol.* **160**, 5203–5208 (1998).
26. Serre, K. *et al.* Efficient presentation of multivalent antigens targeted to various cell surface molecules of dendritic cells and surface Ig of antigen-specific B cells. *J. Immunol.* **161**, 6059–6067 (1998).
27. Green, S. M., Lowe, A. D., Parrington, J. & Karn, J. Transformation of growth factor-dependent myeloid stem cells with retroviral vectors carrying c-myc. *Oncogene* 737–751 (1989).
28. Russell, D. M. *et al.* Peripheral deletion of self-reactive B cells. *Nature* **354**, 308–311 (1991).
29. Goodnow, C. C. *et al.* Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature* **334**, 676–682 (1988).
30. Aluvihare, V. R., Khamlichi, A. A., Williams, G. T., Adorini, L. & Neuberger, M. S. Acceleration of intracellular targeting of antigen by the B-cell antigen receptor: importance depends on the nature of the antigen-antibody interaction. *EMBO J.* **16**, 3553–3562 (1997).

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Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells

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RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene[1–4]. The mediators of sequence-specific messenger RNA degradation are 21- and 22-nucleotide small interfering RNAs (siRNAs) generated by ribonuclease III cleavage from longer dsRNAs[5–9]. Here we show that 21-nucleotide siRNA duplexes specifically suppress expression of endogenous and heterologous genes in different mammalian cell lines, including human embryonic kidney (293) and HeLa cells. Therefore, 21-nucleotide siRNA duplexes provide a new tool for studying gene function in mammalian cells and may eventually be used as gene-specific therapeutics.

Uptake of dsRNA by insect cell lines has previously been shown to 'knock-down' the expression of specific proteins, owing to sequence-specific, dsRNA-mediated mRNA degradation[6,10–12]. However, it has not been possible to detect potent and specific RNA interference in commonly used mammalian cell culture systems, including 293 (human embryonic kidney), NIH/3T3 (mouse fibroblast), BHK-21 (Syrian baby hamster kidney), and CHO-K1 (Chinese hamster ovary) cells, applying dsRNA that varies in size between 38 and 1,662 base pairs (bp)[10,12]. This apparent lack of RNAi in mammalian cell culture was unexpected, because RNAi exists in mouse oocytes and early embryos[13,14], and because RNAi-related, transgene-mediated co-suppression was also observed in cultured Rat-1 fibroblasts[15]. But it is known that dsRNA in the cytoplasm of mammalian cells can trigger profound physiological

reactions that lead to the induction of interferon synthesis[16]. In the interferon response, dsRNA > 30 bp binds and activates the protein kinase PKR[17] and 2′,5′-oligoadenylate synthetase (2′,5′-AS)[18]. Activated PKR stalls translation by phosphorylation of the translation initiation factors eIF2α, and activated 2′,5′-AS causes mRNA degradation by 2′,5′-oligoadenylate-activated ribonuclease L. These responses are intrinsically sequence-nonspecific to the inducing dsRNA.

Base-paired 21- and 22-nucleotide (nt) siRNAs with overhanging 3′ ends mediate efficient sequence-specific mRNA degradation in lysates prepared from *Drosophila* embryos[9]. To test whether siRNAs are also capable of mediating RNAi in cell culture, we synthesized 21-nt siRNA duplexes with symmetric 2-nt 3′ overhangs directed against reporter genes coding for sea pansy (*Renilla reniformis*, RL) and two sequence variants of firefly (*Photinus pyralis*, GL2 and GL3) luciferases (Fig. 1a, b). The siRNA duplexes were co-transfected with the reporter plasmid combinations pGL2/pRL or pGL3/pRL, into *Drosophila* S2 cells or mammalian cells using cationic liposomes. Luciferase activities were determined 20 h after transfection. In *Drosophila* S2 cells (Fig. 2a and b), the specific inhibition of luciferases was complete and similar to results previously obtained for longer dsRNAs[6,10,12,19]. In mammalian cells, where the reporter genes were 50- to 100-fold more strongly expressed, the specific suppression was less complete (Fig. 2c–j). In NIH/3T3, monkey COS-7 and Hela S3 cells (Fig. 2c–h), GL2 expression was reduced 3- to 12-fold, GL3 expression 9- to 25-fold, and RL expression 2- to 3-fold, in response to the cognate siRNAs. For 293 cells, targeting of RL luciferase by RL siRNAs was ineffective, although GL2 and GL3 targets responded specifically (Fig. 2i and j). The lack of reduction of RL expression in 293 cells may be because of its expression, 5- to 20-fold higher than any other mammalian cell line tested and/or to limited accessibility of the target sequence due to RNA secondary structure or associated proteins. Nevertheless, specific targeting of GL2 and GL3 luciferase by the cognate siRNA duplexes indicated that RNAi is also functioning in 293 cells.

The 2-nucleotide 3′ overhang in all siRNA duplexes was composed of (2′-deoxy) thymidine, except for uGL2, which contained

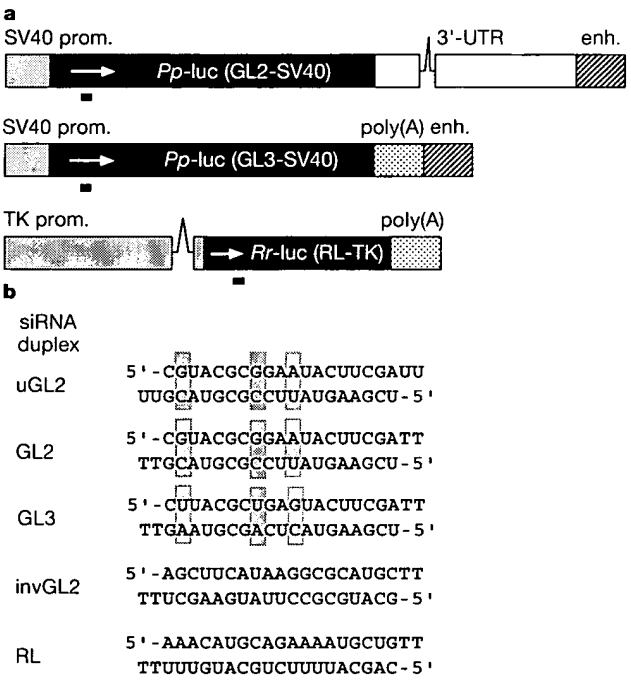

Figure 1 Reporter constructs and siRNA duplexes. **a**, The firefly (*Pp*-luc) and sea pansy (*Rr*-luc) luciferase reporter-gene regions from plasmids pGL2-Control, pGL3-Control, and pRL-TK (Promega) are illustrated; simian virus 40 (SV40) promoter (prom.); SV40 enhancer element (enh.); SV40 late polyadenylation signal (poly(A)); herpes simplex virus (HSV) thymidine kinase promoter, and two introns (lines) are indicated. The sequence of GL3 luciferase is 95% identical to GL2, but RL is completely unrelated to both. Luciferase expression from pGL2 is approximately 10-fold lower than from pGL3 in transfected mammalian cells. The region targeted by the siRNA duplexes is indicated as black bar below the coding region of the luciferase genes. **b**, The sense (top) and antisense (bottom) sequences of the siRNA duplexes targeting GL2, GL3, and RL luciferase are shown. The GL2 and GL3 siRNA duplexes differ by only three single-nucleotide substitutions (boxed in grey). As nonspecific control, a duplex with the inverted GL2 sequence, invGL2, was synthesized. The 2-nucleotide 3′ overhang of 2′-deoxythymidine is indicated as TT; uGL2 is similar to GL2 siRNA but contains ribo-uridine 3′ overhangs.

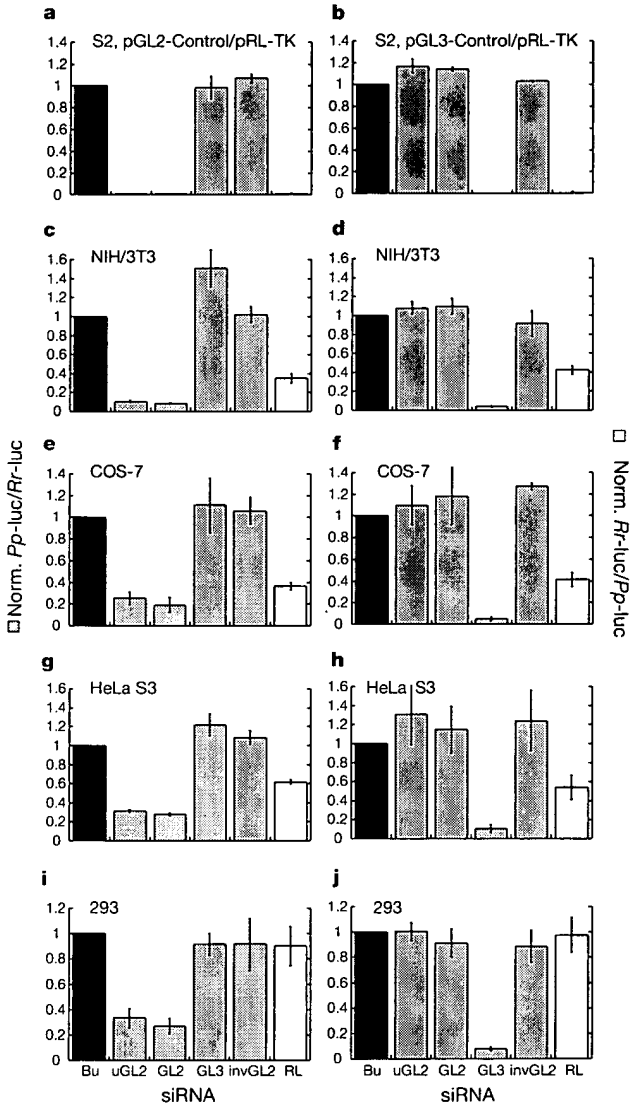

Figure 2 RNA interference by siRNA duplexes. Ratios of target to control luciferase were normalized to a buffer control (Bu, black bars); grey bars indicate ratios of *Photinus pyralis* (*Pp*-luc) GL2 or GL3 luciferase to *Renilla reniformis* (*Rr*-luc) RL luciferase (left axis), white bars indicate RL to GL2 or GL3 ratios (right axis). **a**, **c**, **e**, **g** and **i**, Experiments performed with the combination of pGL2-Control and pRL-TK reporter plasmids; **b**, **d**, **f**, **h** and **j**, experiments performed with the combination of pGL3-Control and pRL-TK reporter plasmids. The cell line used for the interference experiment is indicated at the top of each plot. The ratios of *Pp*-luc/*Rr*-luc for the buffer control (Bu) varied between 0.5 and 10 for pGL2/pRL, and between 0.03 and 1 for pGL3/pRL, respectively, before normalization and between the various cell lines tested. The plotted data were averaged from three independent experiments ± s.d.

uridine residues. The thymidine overhang was chosen because it reduces costs of RNA synthesis and may enhance nuclease resistance of siRNAs in the cell culture medium and within transfected cells. As in the *Drosophila in vitro* system (data not shown), substitution of uridine by thymidine in the 3′ overhang was well tolerated in cultured mammalian cells (Fig. 2a, c, e, g and i), and the sequence of the overhang appears not to contribute to target recognition[9].

In co-transfection experiments, 25 nM siRNA duplexes were used (Figs 2 and 3; concentration is in respect to the final volume of tissue culture medium). Increasing the siRNA concentration to 100 nM did not enhance the specific silencing effects, but started to affect transfection efficiencies, perhaps due to competition for liposome encapsulation between plasmid DNA and siRNA (data not shown). Decreasing the siRNA concentration to 1.5 nM did not reduce the specific silencing effect (data not shown), even though the siRNAs were now only 2- to 20-fold more concentrated than the DNA plasmids; the silencing effect only vanishes completely if the siRNA concentration was dropped below 0.05 nM. This indicates that siRNAs are extraordinarily powerful reagents for mediating gene silencing, and that siRNAs are effective at concentrations that are several orders of magnitude below the concentrations applied in conventional antisense or ribozyme gene-targeting experiments[20].

To monitor the effect of longer dsRNAs on mammalian cells, 50- and 500-bp dsRNAs that are cognate to the reporter genes were prepared. As a control for nonspecific inhibition, dsRNAs from humanized GFP (hG)[21] was used. In these experiments, the reporter plasmids were co-transfected with either 0.21 μg siRNA duplexes or 0.21 μg longer dsRNAs. The siRNA duplexes only reduced the expression of their cognate reporter gene, while the longer dsRNAs strongly and nonspecifically reduced reporter-gene expression. The effects are illustrated for HeLa S3 cells as a representative example (Fig. 3a and b). The absolute luciferase activities were decreased nonspecifically 10- to 20-fold by 50-bp dsRNA, and 20- to 200-fold by 500-bp dsRNA co-transfection, respectively. Similar nonspecific effects were observed for COS-7 and NIH/3T3 cells. For 293 cells, a 10- to 20-fold nonspecific reduction was observed only for 500-bp dsRNAs. Nonspecific reduction in reporter-gene expression by dsRNA > 30 bp was expected as part of the interferon response[16]. Interestingly, superimposed on the nonspecific interferon response, we detect additional sequence-specific, dsRNA-mediated silencing. The sequence-specific silencing effect of long dsRNAs, however, became apparent only when the relative reporter-gene activities were normalized to the hG dsRNA controls (Fig. 3c). Sequence-specific silencing by 50- or 500-bp dsRNAs reduced the targeted reporter-gene expression by an additional 2- to 5-fold. Similar effects were also detected in the other three mammalian cell lines tested (data not shown). Specific silencing effects with dsRNAs (356–1,662 bp) were previously reported in CHO-K1 cells, but the amounts of dsRNA required to detect a 2- to 4-fold specific reduction were about 20-fold higher than in our experiments[12]. Also, CHO-K1 cells appear to be deficient in the interferon response. In another report, 293, NIH/3T3 and BHK-21 cells were tested for RNAi using luciferase/β-galactosidase (lacZ) reporter combinations and 829-bp specific lacZ or 717-bp nonspecific green fluorescent protein (GFP) dsRNA[10]. The lack of detected RNAi in this case may be due to the less sensitive luciferase/lacZ reporter assay and the length differences of target and control dsRNA. Taken together, our results indicate that RNAi is active in mammalian cells, but that the silencing effect is difficult to detect if the interferon system is activated by dsRNA > 30 bp.

To test for silencing of endogenous genes, we chose four genes coding for cytoskeletal proteins: lamin A/C, lamin B1, nuclear mitotic apparatus protein (NuMA) and vimentin[27]. The selection was based on the availability of antibodies needed to quantitate the silencing effect. Silencing was monitored 40 to 45 h after transfection to allow for turnover of the protein of the targeted genes. As shown in Fig. 4, the expression of lamin A/C was specifically reduced by the cognate siRNA duplex (Fig. 4a), but not when nonspecific siRNA directed against firefly luciferase (Fig. 4b) or buffer (Fig. 4c) was used. The expression of a non-targeted gene, NuMA, was unaffected in all treated cells (Fig. 4d–f), demonstrating the integrity of the targeted cells. The reduction in lamin A/C proteins was more than 90% complete as quantified by western blotting (Fig. 4j, k). We note that lamin A/C 'knock-out' mice are

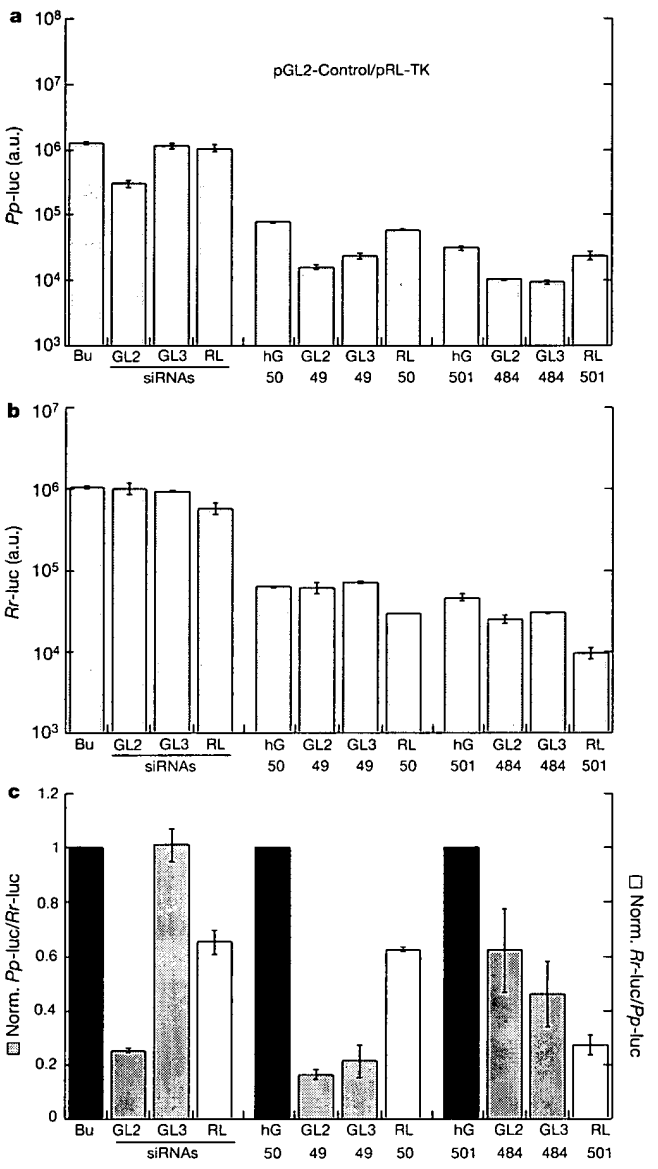

Figure 3 Effects of 21-nucleotide siRNAs, 50-bp, and 500-bp dsRNAs on luciferase expression in HeLa cells. The exact length of the long dsRNAs in base pairs is indicated below the bars. Experiments were performed with pGL2-Control and pRL-TK reporter plasmids. The data were averaged from two independent experiments ± s.d. **a**, Absolute *Pp*-luc expression, plotted in arbitrary luminescence units (a.u.). **b**, *Rr*-luc expression, plotted in arbitrary luminescence units. **c**, Ratios of normalized target to control luciferase. The ratios of luciferase activity for siRNA duplexes were normalized to a buffer control (Bu, black bars); the luminescence ratios for 50- or 500-bp dsRNAs were normalized to the respective ratios observed for 50- and 500-bp dsRNAs from humanized GFP (hG, black bars). We note that the overall differences in sequence between the 49- and 484-bp GL2 and GL3 dsRNAs are not sufficient to confer specificity for targeting GL2 and GL3 targets (43-nucleotide uninterrupted identity in 49-bp segment, 239-nucleotide longest uninterrupted identity in 484-bp segment)[30].

viable for a few weeks after birth[23] and that the lamin A/C knockdown in cultured cells was not expected to cause cell death. Lamin A and C are produced by alternative splicing in the 3′ region and are present in equal amounts in the lamina of mammalian cells (Fig. 4j, k). Transfection of siRNA duplexes targeting lamin B1 and NuMA reduced the expression of these proteins to low levels (data not shown), but we were not able to observe a reduction in vimentin expression. This could be due to the high abundance of vimentin in the cells (several per cent of total cell mass) or because the siRNA sequence chosen was not optimal for targeting of vimentin.

The mechanism of the 21-nucleotide siRNA-mediated interference process in mammalian cells remains to be uncovered, and silencing might occur post-transcriptionally and/or transcriptionally. In *Drosophila* lysate, siRNA duplexes mediate post-transcriptional gene silencing by reconstitution of siRNA-protein complexes (siRNPs), which guide mRNA recognition and targeted cleavage[6,7,9]. In plants, dsRNA-mediated post-transcriptional silencing has also been linked to DNA methylation, which may also be directed by 21-nucleotide siRNAs[24]. Methylation of promoter regions can lead to transcriptional silencing[25], but methylation in coding sequences does not[26]. DNA methylation and transcriptional silencing in mammals are well documented processes[27], yet their mechanisms have not been linked to that of post-transcriptional silencing. Methylation in mammals is predominantly directed towards CpG dinucleotide sequences. There is no CpG sequence in the RL or lamin A/C siRNA, although both siRNAs mediate specific silencing in mammalian cell culture, so it is unlikely that DNA methylation is essential for the silencing process.

Thus we have shown, for the first time, siRNA-mediated gene silencing in mammalian cells. The use of exogenous 21-nucleotide siRNAs holds great promise for analysis of gene function in human cell culture and the development of gene-specific therapeutics. It will also be of interest in understanding the potential role of endogenous siRNAs in the regulation of mammalian gene function. □

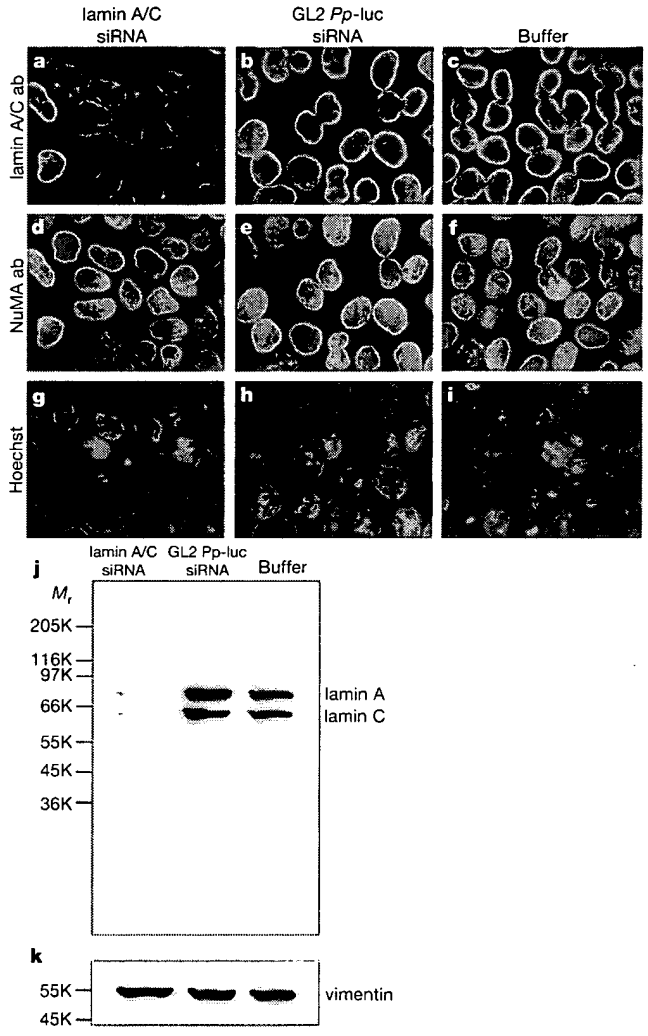

Figure 4 Silencing of nuclear envelope proteins lamin A/C in HeLa cells. Triple fluorescence staining of cells transfected with lamin A/C siRNA duplex (**a**, **d**, **g**), with GL2 luciferase siRNA duplex (nonspecific siRNA control) (**b**, **e**, **h**), and with buffer only (**c**, **f**, **i**). **a**–**c**, Staining with lamin A/C specific antibody; **d**–**f**, staining with NuMA-specific antibody; **g**–**i**, Hoechst staining of nuclear chromatin. Bright fluorescent nuclei in **a** represent untransfected cells. **j**, **k**, Western blots of transfected cells using lamin A/C- (**j**) or vimentin-specific (**k**) antibodies. The Western blot was stripped and re-probed with vimentin antibody to check for equal loading of total protein.

Methods

RNA preparation

21-nucleotide RNAs were chemically synthesized using Expedite RNA phosphoramidites and thymidine phosphoramidite (Proligo, Germany). Synthetic oligonucleotides were deprotected and gel-purified[9]. The accession numbers given below are from GenBank. The siRNA sequences targeting GL2 (Acc. No. X65324) and GL3 luciferase (Acc. No. U47296) corresponded to the coding regions 153–173 relative to the first nucleotide of the start codon; siRNAs targeting RL (Acc. No. AF025846) corresponded to region 119–139 after the start codon. The siRNA sequence targeting lamin A/C (Acc. No. X03444) was from position 608–630 relative to the start codon; lamin B1 (Acc. No. NM_005573) siRNA was from position 672–694; NuMA (Acc. No. Z11583) siRNA from position 3,988–4,010, and vimentin (Acc. No. NM_003380) from position 346–368 relative to the start codon. Longer RNAs were transcribed with T7 RNA polymerase from polymerase chain reaction (PCR) products, followed by gel purification. The 49- and 484-bp GL2 or GL3 dsRNAs corresponded to positions 113–161 and 113–596, respectively, relative to the start of translation; the 50- and 501-bp RL dsRNAs corresponded to position 118–167 and 118–618, respectively. PCR templates for dsRNA synthesis targeting humanized GFP (hG) were amplified from pAD3 (ref. 21), whereby 50- and 501-bp hG dsRNA corresponded to positions 121–170 and 121–621, respectively, to the start codon.

For annealing of siRNAs, 20 μM single strands were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) for 1 min at 90 °C followed by 1 h at 37 °C. The 37 °C incubation step was extended overnight for the 50- and 500-bp dsRNAs, and these annealing reactions were performed at 8.4 μM and 0.84 μM strand concentrations, respectively.

Cell culture

S2 cells were propagated in Schneider's *Drosophila* medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS) 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin at 25 °C. 293, NIH/3T3, HeLa S3, HeLa SS6, COS-7 cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. Cells were regularly passaged to maintain exponential growth. Twenty-four h before transfection at 50–80% confluency, mammalian cells were trypsinized and diluted 1:5 with fresh medium without antibiotics ($1–3 \times 10^5$ cells ml^{-1}) and transferred to 24-well plates (500 μl per well). S2 cells were not trypsinized before splitting. Co-transfection of reporter plasmids and siRNAs was carried out with Lipofectamine 2000 (Life Technologies) as described by the manufacturer for adherent cell lines. Per well, 1.0 μg pGL2-Control (Promega) or pGL3-Control (Promega), 0.1 μg pRL-TK (Promega), and 0.21 μg siRNA duplex or dsRNA, formulated into liposomes, were applied; the final volume was 600 μl per well. Cells were incubated 20 h after transfection and appeared healthy thereafter. Luciferase expression was subsequently monitored with the Dual luciferase assay (Promega). Transfection efficiencies were determined by fluorescence microscopy for mammalian cells lines after co-transfection of 1.1 μg hGFP-encoding pAD3 (ref. 21) and 0.21 μg inverted GL2 siRNA, and were 70–90%. Reporter plasmids were amplified in XL-1 Blue (Stratagene) and purified using the Qiagen EndoFree Maxi Plasmid Kit.

Transfection of siRNAs for targeting endogenous genes was carried out using Oligofectamine (Life Technologies) and 0.84 μg siRNA duplex per well, but it was recently found that as little as 0.01 μg siRNAs per well are sufficient to mediate silencing. HeLa SS6 cells were transfected one to three times in approximately 15 h intervals and were assayed 40 to 45 h after the first transfection. It appears, however, that a single transfection is as efficient as multiple transfections. Transfection efficiencies as determined by immunofluorescence of targeted cells were in the range of 90%. Specific silencing of targeted genes was confirmed by at least three independent experiments.

Western blotting and immunofluorescence microscopy

Monoclonal 636 lamin A/C specific antibody[28] was used as undiluted hybridoma supernatant for immunofluorescence and 1/100 dilution for western blotting. Affinity-purified polyclonal NuMA protein 705 antibody[29] was used at a concentration of 10 μg ml^{-1} for

immunofluorescence. Monoclonal V9 vimentin-specific antibody was used at 1/2,000 dilution. For western blotting, transfected cells grown in 24-well plates were trypsinized and harvested in SDS sample buffer. Equal amounts of total protein were separated on 12.5% polyacrylamide gels and transferred to nitrocellulose. Standard immunostaining was carried out using ECL enhanced chemiluminescence technique (Amersham Pharmacia).

For immunofluorescence, transfected cells grown on glass coverslips in 24-well plates were fixed in methanol for 6 min at −10 °C. Target gene specific and control primary antibody were added and incubated for 80 min at 37 °C. After washing in phosphate buffered saline (PBS), Alexa 488-conjugated anti-rabbit (Molecular Probes) and Cy3-conjugated anti-mouse (Dianova) antibodies were added and incubated for 60 min at 37 °C. Finally, cells were stained for 4 min at room temperature with Hoechst 33342 (1 μM in PBS) and embedded in Mowiol 488 (Hoechst). Pictures were taken using a Zeiss Axiophot camera with a Fluar 40/1.30 oil objective and MetaMorph Imaging Software (Universal Imaging Corporation) with equal exposure times for the specific antibodies.

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1. Fire, A. RNA-triggered gene silencing. *Trends Genet.* **15**, 358–363 (1999).
2. Sharp, P. A. RNA interference 2001. *Genes Dev.* **15**, 485–490 (2001).
3. Hammond, S. M., Caudy, A. A. & Hannon, G. J. Post-transcriptional gene silencing by double-stranded RNA. *Nature Rev. Genet.* **2**, 110–1119 (2001).
4. Tuschl, T. RNA interference and small interfering RNAs. *Chem. Biochem.* **2**, 239–245 (2001).
5. Hamilton, A. J. & Baulcombe, D. C. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**, 950–952 (1999).
6. Hammond, S. M., Bernstein, E., Beach, D. & Hannon, G. J. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404**, 293–296 (2000).
7. Zamore, P. D., Tuschl, T., Sharp, P. A. & Bartel, D. P. RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25–33 (2000).
8. Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363–366 (2001).
9. Elbashir, S. M., Lendeckel, W. & Tuschl, T. RNA interference is mediated by 21 and 22 nt RNAs. *Genes Dev.* **15**, 188–200 (2001).
10. Caplen, N. J., Fleenor, J., Fire, A. & Morgan, R. A. dsRNA-mediated gene silencing in cultured *Drosophila* cells: a tissue culture model for the analysis of RNA interference. *Gene* **252**, 95–105 (2000).
11. Clemens, J. C. *et al.* Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. *Proc. Natl Acad. Sci. USA* **97**, 6499–6503 (2000).
12. Ui-Tei, K., Zenno, S., Miyata, Y. & Saigo, K. Sensitive assay of RNA interference in *Drosophila* and Chinese hamster cultured cells using firefly luciferase gene as target. *FEBS Lett.* **479**, 79–82 (2000).
13. Wianny, F. & Zernicka-Goetz, M. Specific interference with gene function by double-stranded RNA in early mouse development. *Nature Cell Biol.* **2**, 70–75 (2000).
14. Svoboda, P., Stein, P., Hayashi, H. & Schultz, R. M. Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. *Development* **127**, 4147–4156 (2000).
15. Bahramian, M. B. & Zarbl, H. Transcriptional and posttranscriptional silencing of rodent alpha(1) collagen by a homologous transcriptionally self-silenced transgene. *Mol. Cell. Biol.* **19**, 274–283 (1999).
16. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H. & Schreiber, R. D. How cells respond to interferons. *Annu. Rev. Biochem.* **67**, 227–264 (1998).
17. Manche, L., Green, S. R., Schmedt, C. & Mathews, M. B. Interactions between double-stranded RNA regulators and the protein kinase DAI. *Mol. Cell. Biol.* **12**, 5238–5248 (1992).
18. Minks, M. A., West, D. K., Benvin, S. & Baglioni, C. Structural requirements of double-stranded RNA for the activation of 2′,5′-oligo(A) polymerase and protein kinase of interferon-treated HeLa cells. *J. Biol. Chem.* **254**, 10180–10183 (1979).
19. Clemens, M. & Williams, B. Inhibition of cell-free protein synthesis by $pppA^{2'}p^{5'}A^{2'}p^{5'}A$: a novel oligonucleotide synthesized by interferon-treated L cell extracts. *Cell* **13**, 565–572 (1978).
20. Macejak, D. G. *et al.* Inhibition of hepatitis C virus (HCV)-RNA-dependent translation and replication of a chimeric HCV poliovirus using synthetic stabilized ribozymes. *Hepatology* **31**, 769–776 (2000).
21. Kehlenbach, R. H., Dickmanns, A. & Gerace, L. Nucleocytoplasmic shuttling factors including Ran and CRM1 mediate nuclear export of NFAT *In vitro*. *J. Cell. Biol.* **141**, 863–874 (1998).
22. Kreis, T. & Vale, R. *Guidebook to the Cytoskeletal and Motor Proteins*, Parts 2b and 3a (Oxford Univ. Press, Oxford, 1999).
23. Sullivan, T. *et al.* Loss of A-type lamin expression compromises nuclear envelope integrity leading to muscular dystrophy. *J. Cell Biol.* **147**, 913–920 (1999).
24. Wassenegger, M. RNA-directed DNA methylation. *Plant Mol. Biol.* **43**, 203–220 (2000).
25. Mette, M. F., Aufsatz, W., van der Winden, J., Matzke, M. A. & Matzke, A. J. M. Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *EMBO J.* **19**, 5194–5201 (2000).
26. Wang, M.-B., Wesley, S. V., Finnegan, E. J., Smith, N. A. & Waterhouse, P. M. Replicating satellite RNA induces sequence-specific DNA methylation and truncated transcripts in plants. *RNA* **7**, 16–28 (2001).
27. Razin, A. CpG methylation, chromatin structure and gene silencing—a three-way connection. *EMBO J.* **17**, 4905–4908 (1998).
28. Röber, R. A., Gieseler, R. K., Peters, J. H., Weber, K. & Osborn, M. Induction of nuclear lamins A/C in macrophages in *in vitro* cultures of rat bone marrow precursor cells and human blood monocytes, and in macrophages elicited *in vivo* by thioglycollate stimulation. *Exp. Cell Res.* **190**, 185–194 (1990).
29. Harborth, J., Wang, J., Gueth-Hallonet, C., Weber, K. & Osborn, M. Self assembly of NuMA: multiarm oligomers as structural units of a nuclear lattice. *EMBO J.* **18**, 1689–1700 (1999).
30. Parrish, S., Fleenor, J., Xu, S., Mello, C. & Fire, A. Functional anatomy of a dsRNA trigger: Differential requirement for the two trigger strands in RNA Interference. *Mol. Cell* **6**, 1077–1087 (2000).

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Ribosomal peptidyl transferase can withstand mutations at the putative catalytic nucleotide

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Peptide bond formation is the principal reaction of protein synthesis. It takes place in the peptidyl transferase centre of the large (50S) ribosomal subunit. In the course of the reaction, the polypeptide is transferred from peptidyl transfer RNA to the α-amino group of amino acyl-tRNA. The crystallographic structure of the 50S subunit showed no proteins within 18 Å from the active site, revealing peptidyl transferase as an RNA enzyme[1]. Reported unique structural and biochemical features of the universally conserved adenine residue A2451 in 23S ribosomal RNA (*Escherichia coli* numbering) led to the proposal of a mechanism of rRNA catalysis that implicates this nucleotide as the principal catalytic residue[2,3]. *In vitro* genetics allowed us to test the importance of A2451 for the overall rate of peptide bond formation. Here we report that large ribosomal subunits with mutated A2451 showed significant peptidyl transferase activity in several independent assays. Mutations at another nucleotide, G2447, which is essential to render catalytic properties to A2451 (refs 2, 3), also did not dramatically change the transpeptidation activity. As alterations of the putative catalytic residues do not severely affect the rate of peptidyl transfer the ribosome apparently promotes transpeptidation not through chemical catalysis, but by properly positioning the substrates of protein synthesis.

The proposed role of A2451 in the peptidyl transfer reaction is

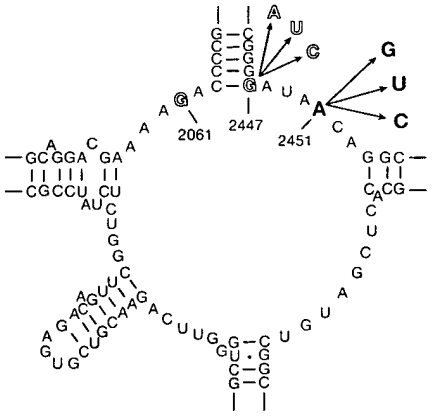

Figure 1 The secondary structure of the central loop of domain V of *T. aquaticus* 23S rRNA. Position A2451 (*E. coli* 23S rRNA numeration), the principal catalytic nucleotide in the proposed general acid–base catalytic mechanism of peptide bond formation[2,3], is shown in bold. Its tertiary interaction partners, guanine residues 2061 and 2447, suggested to be essential for rendering catalytic properties to A2451, are outlined. Arrows indicate the mutations engineered in 23S rRNA.

ATTACHMENT F

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tRNA Genes as Transcriptional Repressor Elements

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Eukaryotic genomes frequently contain large numbers of repetitive RNA polymerase III (pol III) promoter elements interspersed between and within RNA pol II transcription units, and in several instances a regulatory relationship between the two types of promoter has been postulated. In the budding yeast *Saccharomyces cerevisiae*, tRNA genes are the only known interspersed pol III promoter-containing repetitive elements, and we find that they strongly inhibit transcription from adjacent pol II promoters in vivo. This inhibition requires active transcription of the upstream tRNA gene but is independent of its orientation and appears not to involve simple steric blockage of the pol II upstream activator sites. Evidence is presented that different pol II promoters can be repressed by different tRNA genes placed upstream at varied distances in both orientations. To test whether this phenomenon functions in naturally occurring instances in which tRNA genes and pol II promoters are juxtaposed, we examined the sigma and Ty3 elements. This class of retrotransposons is always found integrated immediately upstream of different tRNA genes. Weakening tRNA gene transcription by means of a temperature-sensitive mutation in RNA pol III increases the pheromone-inducible expression of sigma and Ty3 elements up to 60-fold.

Many eukaryotic genomes contain families of moderately to highly repeated DNA elements containing RNA polymerase III (pol III) promoters (reviewed in references 74 and 75). Frequently these elements resemble the intragenic pol III promoter class found in tRNA and 7SL RNA genes, which consist of consensus A-box and B-box sequences downstream from the transcription start sites. These elements can be found either dispersed as individual copies or as highly reiterated tandem copies, especially in heterochromatic regions. The pol III elements are not generally transcribed into stable RNA commensurate with their copy number in vivo, although they can usually be transcribed in vitro, and there are numerous reports of condition-specific or development-specific activation in vivo (10, 27, 61, 90, 95, 97, 101). Several hypotheses have been put forward regarding possible functions for these sequences, but one particularly interesting suggestion is that dispersed RNA pol III promoters might exert either a positive or negative influence on the transcriptional activity of overlapping or nearby RNA pol II promoters (11, 12, 15, 89, 90, 96). In some cases, cryptic pol III promoter elements directly interfere with factor binding sites in the pol II promoter upstream region or with the pol II initiation site itself. In at least one report, however, repression was achieved by an *Alu* repetitive element, in which case there was no obvious steric overlap with the neighboring pol II promoter (96).

In this report, the question of whether RNA pol III promoters can exert negative transcriptional regulation on neighboring DNA has been approached by studying the budding yeast *Saccharomyces cerevisiae*. Although this yeast does not appear to have any *Alu*-type repetitive elements, the repetitive tRNA genes themselves can be considered as a dispersed family of repetitive pol III promoter elements. Yeast cells contain over 400 tRNA genes that occupy over 0.1% of the genome (32), and these genes are frequently found near the upstream control regions for genes transcribed by RNA pol II. In particular, there is a close association of tRNA genes immediately upstream of the Ty retrotransposons (5, 36, 47, 72, 79, 80). This association is precisely positioned in the Ty3 class of full retrotransposon and the more numerous free solo repeats of the sigma repetitive elements that form the Ty3 long terminal repeats. Ty3 insertion occurs with a strong preference near the tRNA gene transcription initiation site (13, 14), and it seems possible that this insertion preference developed because of a regulatory advantage conferred by the neighboring placement on the chromosome. A very similar close association with tRNA genes has been found for the DRE retrotransposons from *Dictyostelium discoideum* (69, 70).

To test whether tRNA genes affect neighboring pol II promoters in yeast cells, we placed several tRNA genes upstream of two entirely different pol II transcription units and found moderate to severe repression of RNA pol II transcription. Further, we present evidence suggesting that transcription of the neighboring tRNA genes plays a role in negatively regulating mating pheromone-responsive expression of chromosomal sigma and Ty3 elements in their normal chromosomal environment.

MATERIALS AND METHODS

Yeast strains and genetic manipulations. Unless specifically stated, plasmid expression results are reported for a strain that is wild type at *GAL4* and *GAL80*, YM607 (*MATa ura3-52 ade2-101 his3-Δ200 lys2-801 trp1-901 GAL4 GAL80*). To test the effects of *gal4* and *gal80* mutations, the related strains YM703 (*MATa ura3-52 ade2-101 his3-Δ200 lys2-801 trp1-901 tyr1-501 GAL4 gal80-Δ538*), YM708 (*MATα ura3-52 ade2-101 trp1-901 gal4-Δ542 GAL80*), and YM709 (*MATa ura3-52 ade2-101 his3-Δ200 lys2-801 trp1-901 tyr1-501 met⁻ gal4-Δ542 gal80-Δ538*) were used. Plasmid constructs were also tested for *SUP4* and *SUP53* tRNA gene and *HIS3* expression in strain YM705 (*MATa ura3-52 ade2-101 his3-Δ200 lys2-801 trp1-901 met⁻ GAL4 GAL80*), unless it is specified that the experiment was also tested in an α strain, YM2062 (*MATα ura3-52 ade2-101 his3-Δ200 lys2-801 tyr1-501 GAL4 GAL80 leu2::GAL1-lacZ*). For testing the effects

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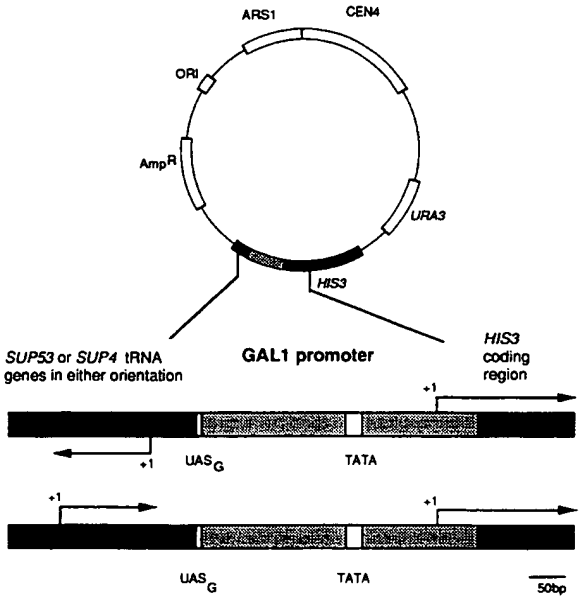

FIG. 1. Juxtaposition of tRNA genes with the UAS_{GAL}/*GAL1* promoter. Expression of *HIS3* coding sequences was put under the control of a consensus UAS_{GAL} and *GAL1* basal promoter as described in Materials and Methods. Transcription of *HIS3* sequences is induced by galactose or actively repressed by dextrose in the growth media (see Fig. 3). *SUP4* and *SUP53* tRNA gene variants were inserted upstream of the UAS_{GAL}, and their effects on transcription from the neighboring pol II promoter in yeast cells were tested by both His phenotype and *HIS3* mRNA levels (Fig. 2 and 3). The approximate extent of the primary transcript from the tRNA gene corresponds to the arrow shown over the tRNA gene.

of temperature-sensitive (ts) mutations in RNA pol III, the parental strain YNN281 (*MATa ura3-52 ade2-101 his3-Δ200 lys2-801 trp1-901*) and the same strain with a ts point mutation in the largest subunit of RNA pol III (YNN281-*rpc160-41*) were used (31, 102). Growth media were prepared and genetic manipulations performed by standard methods (46, 84).

Construction of plasmids. The general constructions of the plasmids used in this study are shown in Fig. 1 and 4. The plasmids in Fig. 1 are derived from a YCp50 variant in which small the *Eco*RI-to-*Hin*dIII fragment of YCp50 was excised and the ends were blunted and religated to remove the original *Eco*RI, *Cla*I, and *Hin*dIII sites. The *GAL1* promoter region (lacking upstream activator sites [UASs]) fused to the *HIS3* coding region was prepared from a previously described clone, p10GH (68), as a *Bam*HI-to-*Sal*I fragment and ligated into the *Bam*HI-to-*Sal*I sites of the modified YCp50. Sequencing of the insert shows that it contains a 270-bp fragment of the *GAL1* promoter (positions 546 to 816 [49]) including the major transcription start site at 760. The upstream end of the fragment was formed by exonuclease III digestion and *Eco*RI linker addition. The downstream end was created by *Xba*I cleavage and filling with DNA polymerase to blunt the site. The *HIS3* fragment was prepared by cleaving pSc2808 with *Eco*RI at −10, blunting the site for ligation to the blunted *Xba*I site of the *GAL1* promoter, and cutting with *Sal*I downstream of the *HIS3* coding sequences for ligation to the vector *Sal*I site. Upstream of the *GAL1* promoter, a single consensus UAS_{GAL} was inserted between the vector *Bam*HI site and the *Eco*RI end of the newly generated *GAL1* promoter, using a double-stranded synthetic oligodeoxynucleotide (top-strand sequence from the *Bam*HI-to-*Eco*RI sites is 5'-GGATCCGGGTGACAGCCCT CCGAAGGAATTC). This vector without tRNA genes is pBM950. tRNA genes were ligated into this *Bam*HI site upstream of the UAS_{GAL}. The *SUP4* tRNA gene variants with 70 bp of downstream sequence and variable amounts of upstream sequence in the *Bam*HI fragments were cloned in both orientations (83). *SUP53* tRNA gene variants with or without internal promoter mutations (43, 73) were amplified by PCR, using primers annealing 60 bp upstream of the start of transcription (−70 to −55; 5'-TCCTTGTTCATGTGTG) or 44 bp downstream of the transcription terminator (GAT TCTGTGCGATAGC). *Bam*HI linkers were added to the fragments, and they were cloned into the *Bam*HI site of pBM950 in both orientations. The sequences of tRNA constructs were confirmed by dideoxynucleotide chain termination sequencing from oligonucleotide primers in the vector, *GAL1*, and tRNA regions.

To make the metallothionein gene (*CUP1*) promoter constructs shown in Fig. 5, *Bam*HI fragments containing the indicated tRNA genes were excised from the pBM950 constructs and placed in either the *Bam*HI site or the *Eco*RV site (after addition of *Bam*HI linkers to the *Eco*RV site, deleting the *Bam*HI-*Eco*RV fragment of the vector) of plasmid pCLUC (gift of Dennis Thiele). pCLUC has, inserted into the *Bam*HI-to-*Eco*RI sites of YCP50, a *Bam*HI-to-*Eco*RI fragment from YIpCL containing the *CUP1* UAS/promoter fused to the β-galactosidase (*lacZ*) coding region as described previously (94). Expression of β-galactosidase activity is induced by the presence of copper in the media.

Assays for expression of the tRNA genes. *SUP4* and *SUP53* are both members of multicopy tRNA gene families (98), and RNA production is therefore difficult to assay directly. Both genes were assayed by phenotype, however. *SUP4* is an ochre suppressor that is tested by suppression of ochre mutations in *ade2* in the strains used for these studies. Constructs were also tested for suppression of the *can1-100* ochre lesion in strain W3031A (*MATa ura3-1 ade2-1 his3-11, 15 trp1-1 can1-100 GAL4 GAL80*; gift of R. Rothstein). All *SUP4* tRNA gene constructs used in this study are expressing *SUP4* by suppression phenotypes. *SUP53* genes were assayed by suppression of the *lys2-801* amber mutation in strain YM705. The *SUP53* gene without promoter mutations was expressed in all orientations and positions shown. As expected (73), all internal promoter mutations (C_{19}, G_{56}, $C_{19}G_{56}$, and $AAA_{10-12}TTT_{23-25}$; positions are denoted by subscript numbers) eliminated the suppression phenotype regardless of the strength of their effect on transcription.

Assays for *HIS3* and *lacZ* expression. Transcription of the *HIS3* gene from the UAS_{GAL}/*GAL1* promoter in the plasmid constructs was assayed qualitatively by two methods to test the effects of either *SUP4* or *SUP53* tRNA gene juxtaposition. First, His^+ phenotype was tested by the ability to grow on solid synthetic medium in the presence of galactose but in the absence of histidine. Relative growth rate (Fig. 2) was estimated by colony size with time when strains were patched or streaked side by side. All *SUP4* tRNA gene insertions shown (Fig. 3) completely prevented *HIS3* expression by this assay, whereas *SUP53* prevented expression and promoter-defective *SUP53* variants allowed different rates of growth. Growth of all constructs in the absence of histidine was tested on repressing (dextrose), noninducing (raffinose), or inducing (galactose plus raffinose) carbon

sources (shown in Fig. 3) to ensure that *HIS3* expression, when present, depended on function of the UAS_{GAL} and *GAL1* promoter. To directly test *HIS3* RNA production, cultures were grown to mid-log phase in synthetic inducing medium (galactose plus raffinose, lacking uracil). Data are shown in Fig. 2 only for the *SUP53* plasmid constructs. RNA was prepared as described previously (59), and 5 μg per lane was subjected to Northern (RNA) blot analysis (78) after separation on 1.5% agarose formaldehyde gels. Hybridization probes were 5'-end-labeled oligonucleotides complementary to the 5' end of the chimeric *HIS3* transcript (5'-GGGCTTTCTGCTCTGTCATCTTTGCC) or the 5' end of the *URA3* mRNA (5'-TGTAGCTTTCGACATG) as an internal control.

Expression of β-galactosidase from the *CUP1* UAS/promoter constructs (Fig. 5) was assayed in triplicate in at least two separate experiments as described previously (30, 35). Although absolute numbers of units varied between experiments, triplicate assays within an experiment were reproducible (±5%), and the degree of repression by tRNA genes was consistent between experiments. Enzyme units, shown for one representative experiment, are expressed as $200 \times (OD_{420}/OD_{600})$, where OD_{420} and OD_{600} represent optical densities at 420 and 600 nm. Cultures of 5 ml in synthetic medium lacking uracil were grown to an OD_{600} of 1.0, 100 mM cupric sulfate was added for 45 min to induce the *CUP1* promoter, and the cells were harvested for assay.

Chromosomal footprinting. Chromosomal footprinting was performed essentially as described previously (45) to detect DNase I sensitivity of the *SUP4-77s* gene and UAS_{GAL} in cellular chromatin compared with DNase I sensitivity of the naked DNA. Briefly, exponential cultures of yeast strains bearing the single-copy plasmids in selective media were harvested, washed, digested briefly with high concentrations of Zymolyase, and lysed hypotonically. Hypotonic lysates were immediately subjected to DNase I digestion for 5 min, and the DNA was purified. Cleavages were detected on both strands by annealing of ^{32}P-labeled oligodeoxynucleotides either 140 bp upstream of the tRNA gene in the plasmid vector or immediately downstream of the UAS and extension with *Taq* DNA polymerase for 15 rounds of annealing and extension. (Choice of primers was constrained by the need to overlap cloning junctions to ensure unique hybridization to only the plasmid constructs and not elsewhere in the yeast genome.) Position markers for the primer extensions were provided by dideoxynucleotide sequencing ladders produced from the same primers. Detailed chromosomal footprint analysis of the tRNA genes and interaction of Gal4 protein with the UAS_{GAL} is described elsewhere (41, 44).

Assays for sigma and Ty3 element induction. Strain YNN281 and its derivative bearing a ts mutation in the large subunit of RNA pol III (YNN281-*rpc160-41*) were grown to early log phase in synthetic complete medium at room temperature (23°C) and shifted to 37°C with prewarmed medium for the indicated length of time. At the indicated time, α mating pheromone (Sigma Chemical) was added to 10 μg/ml, and growth continued for 30 min. Cells were harvested and RNA was prepared as for Northern analysis, and the levels of sigma, Ty3, and *ADH1* RNAs were determined by primer extension on all three types of RNA simultaneously in the same reactions (62). The 5'-^{32}P-labeled primers annealed near the 5' ends of the sigma element consensus (5'-CGAGTAATACCGGA [79]), the Ty3-1 element that is known to be expressed (5'-AGACTCATAA GATGA [16, 34]), and ADH1 (5'-CGTAGAAGATAACAC CT [4]). Primer extension products were separated by electrophoresis on DNA sequencing gels, the gels were dried under vacuum, and the radioactivity in the indicated bands (Fig. 6) was quantitated with a Betascope 603 blot analyzer. Under no conditions were significant levels of sigma or Ty3 RNA detected in the absence of α-factor induction, and only the α-factor-induced RNA is shown. Expression of sigma and Ty3 RNAs was also evaluated relative to a *URA3* internal control by Northern blot analysis (not shown), with qualitatively indistinguishable results. Primer extension results were used for quantitation because multiple sigma-specific bands were present in the small-molecular-weight range of Northern blots, presumably as a result of differences in 3'-end formation of transcripts initiated within the many sigma copies. (We estimate by Southern blot that there are 4 copies of Ty3 and >20 copies of sigma in strain YNN281.) Probes were random-primed DNA from PCR-generated entire sigma fragment or an internal *Bgl*II fragment of Ty3-1 (34).

RESULTS

Repression of pol II promoters by upstream tRNA genes. To approach the question of interference between neighboring pol II and pol III promoters, we juxtaposed tRNA genes and pol II transcription units that could be assayed phenotypically for expression. We chose two entirely distinct tRNA genes: the *SUP53* amber suppressor variant of a $tRNA^{Leu}$ gene family (1, 73) and the *SUP4* ochre suppressor of a $tRNA^{Tyr}$ gene family (28, 60, 83). These genes were inserted upstream of two different pol II-specific promoters. The first of these plasmid constructs is depicted in Fig. 1. The pol II transcription unit consists of the yeast *HIS3* coding region fused to the *GAL1* promoter, with a single consensus Gal4 protein binding site (UAS_{GAL} or UAS_G) upstream of the basal promoter. In this construct, *HIS3* expression, and therefore the ability to grow in the absence of histidine, depends on Gal4 protein. Cells were always His^- in media containing dextrose, since Gal4 protein is inactive under this condition; expression of *HIS3* was tested in medium containing galactose, in which Gal4 protein is active. Expression of the various tRNA genes inserted upstream of the UAS_G was tested phenotypically by suppression of an amber *lys2* allele in the case of *SUP53* and by suppression of ochre *ade2* and *can1* alleles in the case of *SUP4*. On growth media not selective for *HIS3*, *SUP4*, or *SUP53*, the yeast strains grew equally well regardless of which pol II or pol III promoter construct was present on the plasmid.

Figure 2 shows the results of inserting the *SUP53* tRNA gene and variants with previously characterized point mutations in the A-box and B-box internal promoters (41, 73). The effects of inserting the tRNA genes were qualitatively the same regardless of their orientation (not shown). The expressed *SUP53* gene with a wild-type promoter abolished growth in the absence of histidine (Fig. 2A) and reduced *HIS3* RNA expression (Fig. 2B). However, mutations in the tRNA gene internal promoter alleviated this repression to a degree roughly commensurate with the severity of the effect of the mutations on transcription. It is interesting that promoter mutations in both the A box and B box alleviate repression. The B-box mutations (C_{56} and $G_{19}C_{56}$) completely block binding of the initial transcription assembly factor, TFIIIC, in vitro and in vivo (41, 73), which prevents all further complex formation on the genes (41). In contrast, the A-box mutations alone decrease transcription by interfering with upstream transcription initiation complex forma-

A

UAS_G	*SUP53* tRNA gene variant[1]	tRNA gene promoter strength[2]	*HIS3* expression[3] dex[4]	gal[4]
-	-	-	-	-
+	SUP53 (WT)	100%	-	-
+	$AAA_{10-12}TTT_{23-25}$ (no A box)	10%	-	+
+	C_{19} (no A box)	10%	-	+
+	G_{56} (no B box)	5%	-	++
+	$C_{19}G_{56}$ (no A or B box)	<1%	-	++
+	-	-	-	+++

[1]-50 bp of 5' flanking sequence; same result in either orientation
[2]-from Newman *et. al.*, 1983
[3]-growth in media lacking histidine
[4]-dextrose or galactose carbon source

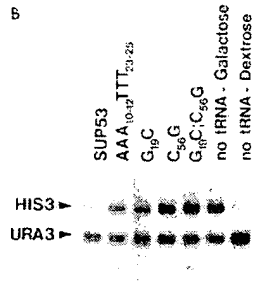

FIG. 2. Effects of the *SUP53* tRNA gene on neighboring expression of *HIS3*. The *SUP53* tRNA gene and four internal promoter variants with different degrees of residual transcription activity (73) were inserted in both orientations upstream of the UAS_{GAL} as shown in Fig. 1 and tested for their effects on *HIS3* expression. The *SUP53* gene fragments contained 60 bp upstream of the transcription initiation site and 44 bp downstream of the transcription terminator. Both orientations of a given tRNA gene variant gave the same results. (A) *HIS3* expression from the *GAL1* promoter (with or without the UAS_G) in either inducing (galactose) or repressing (dextrose) medium was determined by relative growth phenotypes in medium lacking histidine. (B) RNA from yeast cells containing the same plasmids as in panel A was subjected to Northern analysis with simultaneous probes to *HIS3* mRNA and *URA3* mRNA as an internal control. The two right-hand lanes show *HIS3* expression from the plasmid without a tRNA gene insert under inducing (galactose) and repressing (dextrose) conditions. RNAs in all other lanes are from cells grown under inducing conditions. Data are shown only for tRNA gene fragments inserted in the transcriptional orientation opposite that of the UAS_{GAL}/*GAL1* promoter.

tion but do not prevent binding of TFIIIC to the internal promoter B box. This finding suggests that it is not TFIIIC binding that results in repression of the neighboring pol II promoter. The ability of the tRNA gene to repress in both transcriptional orientations (see also Fig. 3) is also noteworthy. It suggests that the repression mechanism does not involve pol III readthrough transcription stripping bound complexes from the pol II promoter sites (see Discussion). It also makes it seem less likely that pol II repression results from direct steric interference by any particular protein components bound to either the upstream or downstream side of the tRNA gene transcription complex.

To reduce further the chances that some DNA sequences extraneous to the tRNA gene contribute to the repression phenomenon, an unrelated tRNA gene, *SUP4*, was also tested. Figure 3 summarizes phenotype assays for *SUP4* tRNA gene expression (scored by ochre suppression) and *HIS3* expression (scored by growth in medium lacking histine) in various genetic backgrounds with respect to the *GAL4* and *GAL80* transcriptional regulators. In addition, all constructs were tested under repressing, inducing, and noninducing growth conditions for Gal4 protein-mediated transcription. In the absence of a tRNA gene, the predicted phenotypes were obtained. A functional Gal4 protein was required for *HIS3* expression to be detected, and this also required induction with galactose unless negative regulation by *GAL80* was alleviated. With a *gal80* mutant, weak growth in the absence of histidine was obtained on a noninducing carbon source (*GAL4*$^+$ *GAL80*$^-$, Raf column) but not with a repressing carbon source (Dex column). In the presence of a *SUP4* tRNA gene insert, however, the tRNA gene was always expressed and *HIS3* was not expressed.

As indicated in Fig. 3, *SUP4* was inserted in both orientations upstream of the UAS_{GAL}, with various amounts of upstream flanking sequence to buffer the UAS_{GAL} from the tRNA transcription unit. Since the required yeast tRNA gene promoter sequences are contained entirely within the coding sequences, it was expected that even deletion of all 5' sequences down to position −4 (−4*o* and −4*s* in Fig. 3) would allow expression of the tRNA gene. In the case of −4*o*, the pol III preinitiation complexes and TFIIIB binding are likely to be in direct competition with Gal4 protein binding to the UAS_{GAL}, which may have been expected to inhibit tRNA transcription (22, 23). However, for all of the other constructs shown in Fig. 3, it is unlikely that there is direct steric overlap between components of the tRNA gene transcription machinery and Gal4 protein. We have previously examined the DNase I footprints of both tRNA gene complexes and Gal4 protein complexes in chromatin (41, 43, 44, 45). The tRNA gene complexes extend from 50 bp upstream of the tRNA coding sequences to 10 bp downstream of the coding sequences. This is in good agreement with steric limits of such complexes in vitro (52, 54), and the positions of factors and RNA pol III within the complex have been assigned (see models in Fig. 7). Gal4 protein-dependent complexes lead to a much smaller protected region in chromatin footprinting (44), only about 20 bp centered on the UAS sequences.

Complexes formed in vivo on the *SUP4*-77*s* construct (Fig. 3) were probed by chromatin footprinting of both strands, and the results are shown in Fig. 4. Both the full expected footprint from the tRNA gene complex and a strong footprint over the UAS_{GAL} are clearly visible, with over 40 bp of DNase I-accessible DNA separating the two regions on both strands. Since these footprints were done under conditions in which growth in galactose failed to induce *HIS3* expression in −77*s*, the tRNA gene is probably not acting by blocking the binding of the Gal4 protein to the UAS_{GAL} but rather is acting at some subsequent step in activation (see Discussion).

To rule out the possibility that repression of pol II expression was some phenomenon peculiar to the *GAL* regulatory

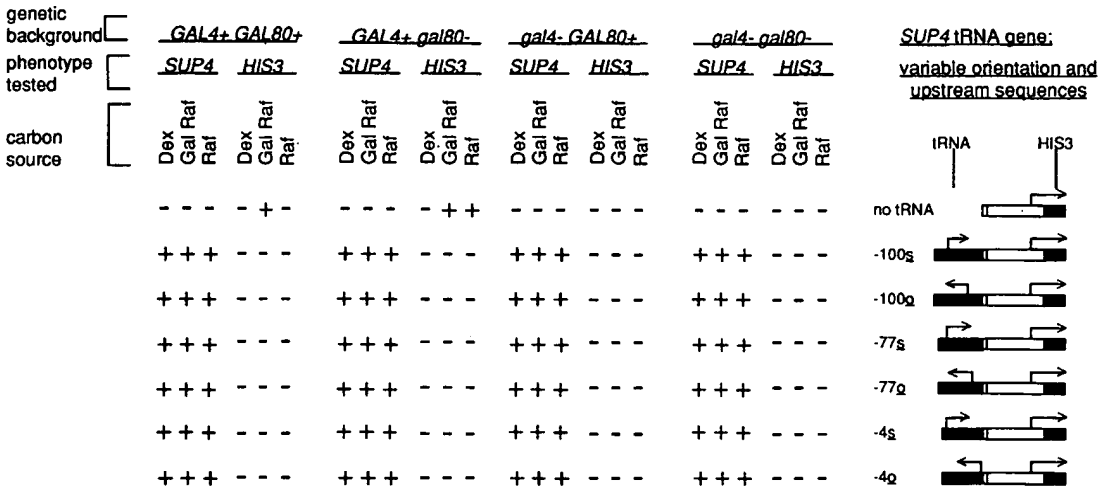

FIG. 3. Insertion of a SUP4 tRNA gene: effects of tRNA gene flanking region and *GAL* regulatory protein mutations. An ochre suppressor *SUP4* tRNA gene with 70 bp of native downstream flanking sequence and between 4 and 100 bp of upstream flanking sequence was inserted upstream of the UAS_{GAL} in the constructs shown in Fig. 1. Individual constructs are indicated at the right. tRNA gene fragments were inserted either in the same (*s*) or opposite (*o*) transcriptional orientation as the *GAL1* promoter-*HIS3* fusion gene. Expression of both the *SUP4* tRNA genes and the *HIS3* gene was assayed by growth phenotype. Expression of the tRNA gene allows growth in the absence of adenine (*SUP4* columns) by suppressing an *ade2* ochre mutation. Expression of *HIS3* allows growth in the absence of histidine (*HIS3* columns). Different combinations of mutations in the *GAL4* and *GAL80* regulatory protein genes were tested for their effects on promoter interference under conditions that were repressing (Dex [dextrose]), inducing (Gal Raf [galactose plus raffinose]), or noninducing (Raf [raffinose]) for *HIS3* expression from the UAS_{GAL}/*GAL1* promoter construct. Under all conditions in all strains, *HIS3* expression behaved as expected in the absence of a tRNA gene. In the presence of any of the tRNA genes, the tRNA gene was expressed and *HIS3* was repressed.

cascade, selected tRNA genes were also inserted upstream of an entirely unrelated UAS/promoter region, that of the *S. cerevisiae* metallothionein gene, *CUP1*. This promoter is activated by the presence of copper and other metals, which induce binding of the *ACE1* transcription factor to the UAS_{CUP1} in vivo and in vitro (25, 42). This UAS/promoter was fused to the β-galactosidase coding region to provide a reporter pol II gene product that was unrelated to *HIS3*, and tRNA genes were inserted in both orientations either 200 or 40 bp upstream of the UAS_{CUP1} control elements (Fig. 5). The result of this experiment, as judged by β-galactosidase activity induced by copper, is that the presence of either the *SUP4* or *SUP53* tRNA gene in either orientation represses expression from the *CUP1* UAS/promoter by between three- and eightfold. Internal promoter mutations in the *SUP53* tRNA gene ($C_{19}G_{56}$) alleviated this repression. Pol II expression was not as completely blocked as in the *GAL* constructs, which we take as an indication that the degree of repression may depend on the exact type of pol II promoter. Repression, although slightly variable among constructs, appeared to be of roughly the same magnitude whether the tRNA genes were inserted 40 or 200 bp from the nearest *CUP1* transcriptional regulatory site. This finding lends further support to the notion that repression is not due to simple steric blockage of the pol II UAS or promoter site. These results clearly confirm that the repressing effect is not specific for the *GAL* UAS/promoter and suggest that tRNA genes might repress surrounding chromatin in their naturally occurring chromosomal locations.

Repression by tRNA genes in native chromosomal locations. Repression by tRNA genes was documented with the *GAL1* and *CUP1* promoters because their regulation and the factors bound to their UAS sequences are well understood. To test the physiological validity these observations at chromosomal loci, we wanted to examine a naturally occurring case in which pol III and pol II promoters are juxtaposed. One particularly intriguing position in which yeast tRNA genes are often found is immediately upstream of pol II promoters in sigma repetitive elements, both solo sigma elements and those found as long terminal repeats of Ty3 retrotransposons. Although tRNA genes are found upstream of all classes of Ty retrotransposons (5), sigma and Ty3 elements are always found inserted exactly at the transcription initiation site of various tRNA genes in a head-to-head orientation. This appears to reflect a preferential Ty3 insertion mechanism (13, 14, 57), but it is possible that this insertion preference might have developed because the juxtaposition confered some advantage to either the retrotransposon or the organism. For example, one could imagine unrestrained expression of the Ty reverse transcriptase genes being detrimental to the yeast host and conditional repression by the neighboring tRNA gene providing a selective advantage. Since it is possible to induce pol II transcription of sigma and Ty3 elements with α mating pheromone, such repression could not be complete as for the *GAL* UAS/promoter. It might, however, partially repress expression or function only conditionally to prevent promiscuous Ty or sigma transcription.

To test for possible repression of all of the transcribed chromosomal Ty3 and sigma elements by neighboring pol III transcription, we used a conditional mutation in RNA pol III. The mutant strain, ts-*rpc160-41*, bears a ts point mutation in the largest subunit of RNA polymerase III that appears to cause defective enzyme assembly and inhibit pol III transcription starting 6 to 10 h after shift to 37°C (31, 62, 102). The mutant and its parental wild-type strain were shifted from the permissive temperature (23°C) to the nonpermissive temperature (37°C) for various lengths of time

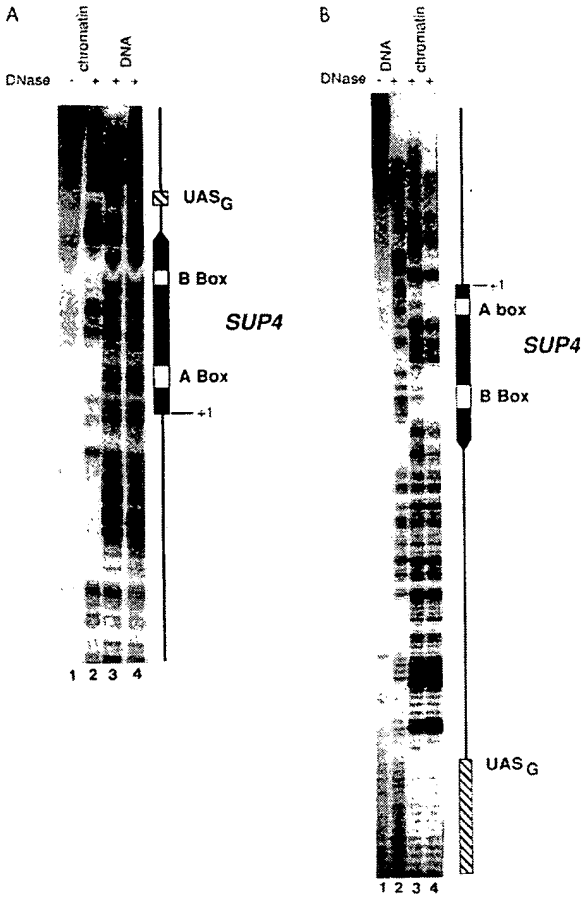

FIG. 4. Chromatin footprinting of the *SUP4* tRNA gene/UAS$_{GAL}$ region. Chromatin footprinting was used to examine complexes stably formed on the UAS$_{GAL}$ and *SUP4-77s* tRNA gene in vivo. Primer extension was used to detect DNase I-sensitive cleavages in chromatin (A, lane 2; B, lanes 3 and 4) in comparison with DNase I digestion of deproteinized DNA (A, lanes 3 and 4; B, lane 2). Primer extensions are also shown on undigested DNA (lanes 1) to indicate primer extension stops independent of cleavage. (A) Cleavage patterns of the template strand; (B) cleavage patterns on the sense strand. A schematic representation to the right of each panel indicates the positions of the tRNA coding sequences (dark arrows) with A-box and B-box internal promoters and the *UAS*$_G$ (striped box). Schematic representations of complexes on the tRNA gene and UAS are shown in Fig. 7. (Duplicate digestions of the naked DNA [A] or chromatin [B] at different DNase I concentrations are shown because the degrees of digestion between the naked DNA and chromatin did not match precisely and the duplicate digestions encompass the exactly equivalent degree of digestion.)

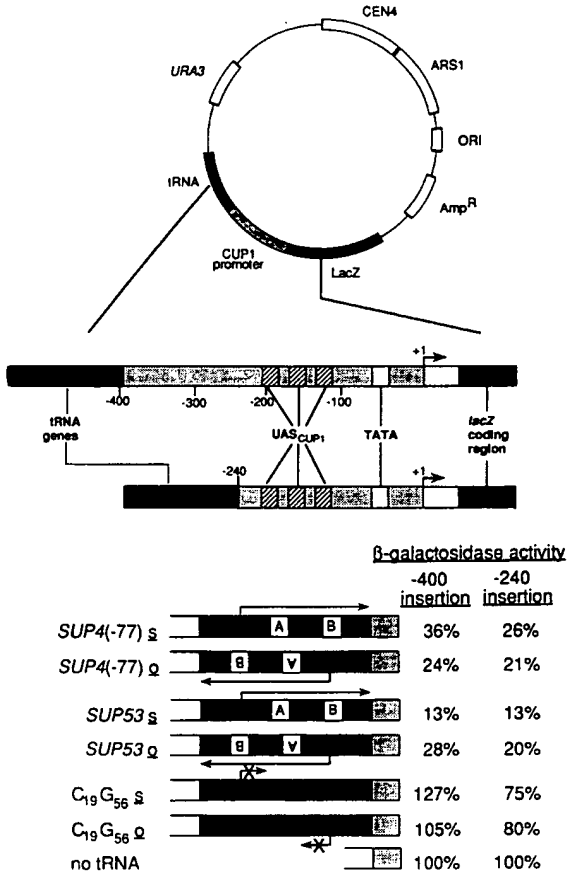

FIG. 5. Effects of tRNA genes flanking a *CUP1* UAS/promoter. The upstream region of the yeast *CUP1* gene was fused to the coding region for β-galactosidase, and various tRNA genes were inserted either 40 bp (−240 insertion) or 200 bp (−400 insertion) upstream of the most distal UAS$_{CUP1}$ transcriptional control element (41). The *CUP1* promoter was induced by addition of copper to the growth media in exponential cultures, and the induction of β-galactosidase activity was measured as described in Materials and Methods. Activity is expressed as a percentage of activity from the construct with no tRNA gene inserted. tRNA genes are the same as in those used in the *GAL* constructs (Fig. 1 to 3).

and then induced with α mating pheromone for 30 min prior to harvest and RNA primer extension analysis. The results of simultaneous primer extensions on total sigma RNAs, Ty3 RNAs, and *ADH1* RNA (internal control for pol II transcription) after α-factor induction are shown in Fig. 6 and expressed as ratios in Table 1. Uninduced sigma and Ty3 RNA levels (not shown) were low and indistinguishable between the wild-type and mutant strains. When both strains were induced after growth at the permissive temperature, induction of sigma and Ty3 RNAs was slightly (less than twofold) but reproducibly elevated in the pol III mutant relative to the wild type. This might be due to mildly defective pol III even at permissive temperatures, since the mutant strain grows slightly more slowly than the parental wild type under these conditions.

A very large difference in sigma and Ty3 expression between the wild-type and pol III ts strain was seen at the nonpermissive temperature, however, and this difference was due primarily to the fact that only the wild-type strain became uninducible by mating pheromone at 37°C. To test the full effect of the pol III mutation, a time course of temperature shift before induction was required. Unexpectedly, the parental wild-type strain consistently became uninducible by α factor, starting less than 6 h after the temperature shift and becoming almost completely uninducible by 12 h. It is not clear why the sigma and Ty3 elements

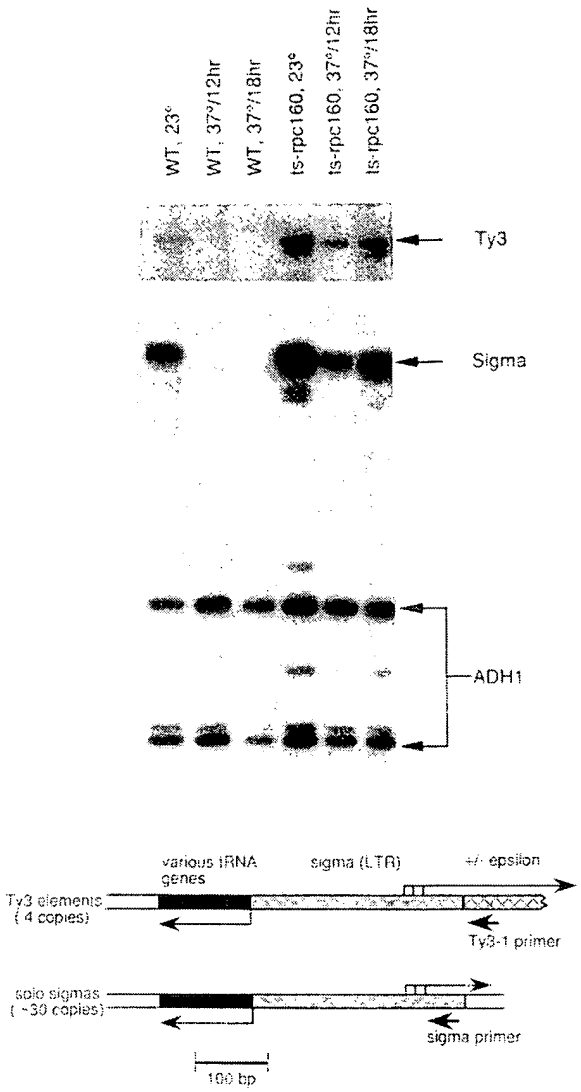

FIG. 6. Effects of a ts pol III mutation on sigma/Ty3 element induction. A yeast strain with a ts point mutation in the large subunit of RNA polymerase III (ts-rpc160) and the parental wild-type strain (WT) were initially grown to early log phase at 23°C and then shifted to 37°C to continue growing for the indicated lengths of time to allow phenotypic manifestations of the ts mutation (see Materials and Methods). Transcription from the solo sigma elements and Ty3 elements was then induced with α mating pheromone for 30 min. RNA was prepared and analyzed by primer extension using radiolabeled oligonucleotides specific for *ADH1* mRNA (internal control), sigma RNA, and Ty3 mRNA in the same primer extension reactions. The positions to which the primer probes hybridized to the consensus sigma sequence and expressed Ty3-1 sequence are indicated at the bottom on a schematic representation of the ubiquitous association of sigma and Ty3 with various tRNA genes. In the absence of α mating pheromone induction, sigma and Ty3 signals were insignificant under all growth conditions (not shown). LTR, long terminal repeat.

become uninducible, but the contrast with the pol III ts mutants suggest that the mechanism requires pol III transcription. Ty3 and sigma expression in the pol III ts mutant starts to lose inducibility at 6 h, similar to the case for the parental wild type. However, the pol III ts strain regains partial inducibility by 12 h and almost full inducibility by 18 h, when the pol III defect is starting to have pronounced effects on tRNA expression (62).

It is formally possible that some product of RNA pol III transcription is required in *trans* for repression of the sigma and Ty3 elements at elevated temperatures. A more straightforward interpretation in light of the preceding *GAL* and *CUP1* promoter data, however, is that this conditional sigma/Ty3 repression requires active transcription of the adjacent tRNA genes.

DISCUSSION

Mechanism of repression by tRNA genes. Multiple forms of negative transcriptional regulation have been characterized in eukaryotes (discussed in reference 64), including inactivation or sequestration of positive regulators, binding of repressor proteins, transcriptional competition, and silencing (6, 18, 39). It is not entirely clear which of these categories, if any, apply to the negative effect tRNA genes exert on neighboring RNA pol II transcription units, but several types of interference between the transcription units appear to be ruled out.

It seems unlikely that either readthrough by pol III or positive supercoils propagated in front of the transcribing pol III are disrupting the pol II UAS or promoter complexes. This is primarily inferred from the fact that the tRNA genes repress in both orientations, although it is formally possible that the mechanism of repression is different for the two orientations. Direct steric interference with binding of pol II transcription factors to the UAS elements and promoters is also improbable for several reasons. Not only do the tRNA genes repress at considerable distance from the pol II UAS elements, but direct examination of the chromatin showed that the UAS is occupied, presumably by Gal4 protein (44), when pol II transcription is repressed by the tRNA gene. The ability of the tRNA genes to repress in both orientations also argues against direct occlusion of the UAS, since different ends of the tRNA complex would have to be involved in the two cases.

Although derepression of Ty3/sigma expression by the pol III ts mutant suggests that the negative regulatory component might be (or act through) pol III itself, there is no direct evidence for this. Other components that interact with the polymerase and would therefore be indirectly affected by a pol III mutation should be considered. Similarities between the upstream elements of pol II and pol III promoters and the factors that help to recognize them suggest that there might be crossover interactions between pol II and pol III components (58, 65, 71, 86, 99, 100). One intriguing idea is that the TFIIIB factor, which binds immediately upstream of the pol III transcription initiation site, might be inhibiting pol II transcription by serving as a competitive ligand for components of the pol II transcription machinery. TFIIIB is composed of several subunits, including the TATA-binding protein (3, 40, 50, 53, 66, 67, 92, 103, 104). If this factor, or any other component of the pol III complex (7, 9, 17, 19, 21, 48, 51, 63, 91, 105), bound tightly to the activation domains of factors bound at the UAS elements, interactions with the pol II basal promoter complex could be competed for (Fig. 7, model A). Similarly, some component of the pol III complex

TABLE 1. Quantitation of sigma and Ty3 RNAs[a]

Construct	σ/*ADH*	Ty3/*ADH*	$(\sigma/ADH)_{mut}/(\sigma/ADH)_{WT}$	$(Ty3/ADH)_{mut}/(Ty3/ADH)_{WT}$
WT				
23°C	2.52	0.68		
37°C				
12 h	0.57	0.17		
18 h	0.07	0.07		
ts-*rpc160*				
23°C	5.11	0.88		
37°C				
12 h	0.74	0.20		
18 h	4.23	1.00		
ts-*rpc160*/WT				
23°C			2.01	1.29
37°C				
12 h			1.30	1.18
18 h			60.4	14.3

[a] The amount of radiolabel in the sigma and Ty3 primer extension bands in Fig. 6 is expressed as a ratio to the *ADH1* internal control for the wild-type parent (WT) and mutant ts-*rpc160*. The derepression of sigma and Ty3 RNA signals by the pol III ts lesion is also expressed as a ratio of the mutant (mut) signal to the wild-type signal.

might directly interact with the pol II basal promoter elements and either prevent productive interactions with upstream factors bound at the UAS (model B) or sterically block some aspect of assembly of the pol II complex (model C). The pol III complex might also attract the binding of an abundant general repressor (56) that is not yet apparent in our footprinting studies.

A last major category of repression mechanism might be modification of chromatin structure by the tRNA gene transcription complex (model D) (8) and could be thought of more generally as attracting unknown repressors to the region of DNA. While we currently have no evidence that tRNA genes order local chromatin structure, phased nucleosomes have been found associated with chromosomal tRNA and 5S rRNA genes (20, 24, 93). Such nucleosome arrays could specifically obscure pol II transcription signals bidirectionally at considerable distance from the tRNA gene, making the tRNA gene seem like a silencer element. Silencers, like enhancers, are defined as being orientation independent and reasonably distance independent, whereas repressor action is normally defined as position-specific binding that sterically interferes with some step in initiation complex assembly. Silencing at the two silent mating-type loci in *S. cerevisiae* has been studied extensively and requires both sequence-specific DNA-binding proteins and modulation of local chromatin structure (55; reviewed in reference 37). Repression of transcription near telomeres and at specific promoters (e.g., *PHO5*) has also been observed to involve chromatin structure in yeast cells (2, 26, 29, 33, 38, 76, 77, 81, 85, 87, 88), although the mechanisms by which these effects occur and their relation to silencing are not yet known. We have avoided the use of the term "silencer" in referring to repression caused by tRNA genes in yeast cells, at least until more is known of the mechanisms in both cases. In considering the relationship between the two, it is worth noting that the silencer at a yeast silent mating-type locus is dominant over (represses) transcription of a tRNA gene at the locus (82).

Possible roles for repression by tRNA genes. It seems certain that tRNA genes did not originally evolve to function as pol II transcriptional repressor elements, yet it is not unreasonable that the cell might use the repressing properties of a pol III promoter as one regulatory aspect of the chromosomal context of a pol II transcription unit. Many tRNA genes in yeast cells are repetitive, and eukaryotes with larger genomes tend to have both dispersed repetitive elements with tRNA-like promoters and cryptic pol III promoter elements distributed in and around pol III transcription units. Whatever forces gave rise to these repeated pol III elements and determined their positioning in chromosomes, there have been suggestions in the literature that they affect nearby pol II transcription. Our current data show that the presence of a tRNA gene can strongly repress nearby pol II transcription but that the degree of repression and conditions under which it manifests are variable.

The *GAL1* and *CUP1* promoters, controlled by quite different UAS-binding proteins, are both subject to this repression under all growth conditions tested, but it is not clear that the chromosomal sigma and Ty3 element promoters are as strongly affected by their associated tRNA genes at moderate growth temperatures. We are able to demonstrate complete repression of α-factor-induced sigma/Ty3 transcription only when cells are grown at elevated temperature. The fact that this repression is alleviated by inactivation of RNA pol III suggests the neighboring tRNA gene is involved in the repression mechanism. There are also many other instances in which yeast tRNA genes naturally occur upstream of pol II transcription units that can clearly be expressed (e.g., the *LEU2* gene [1]). It is not known, however, whether the tRNA genes contribute to the regulation of pol II transcription units in a more subtle fashion than the constructs tested here do. As noted above, both the type of pol II promoter and other determinants of the chromatin context could be dominant over the tRNA effects.

It is not clear what relationship the observed transcriptional repression by yeast tRNA genes might have to tRNA-like promoter elements distributed throughout the genomes of larger organisms. It seems unlikely that the pol III promoters act as repressors in all or even most instances, especially since they appear transcriptionally inert in most tissues examined. However, these repeated sequences have the capacity to bind pol III transcription factors and be transcribed in vitro, and a subset of these sequences might be activated in vivo, whether or not stable RNA products accumulate (10, 27, 61, 90, 95, 101). Given this potential, it would not be surprising to find that the pol III promoters contribute to the transcriptional regulation of the surrounding chromatin in many different contexts.

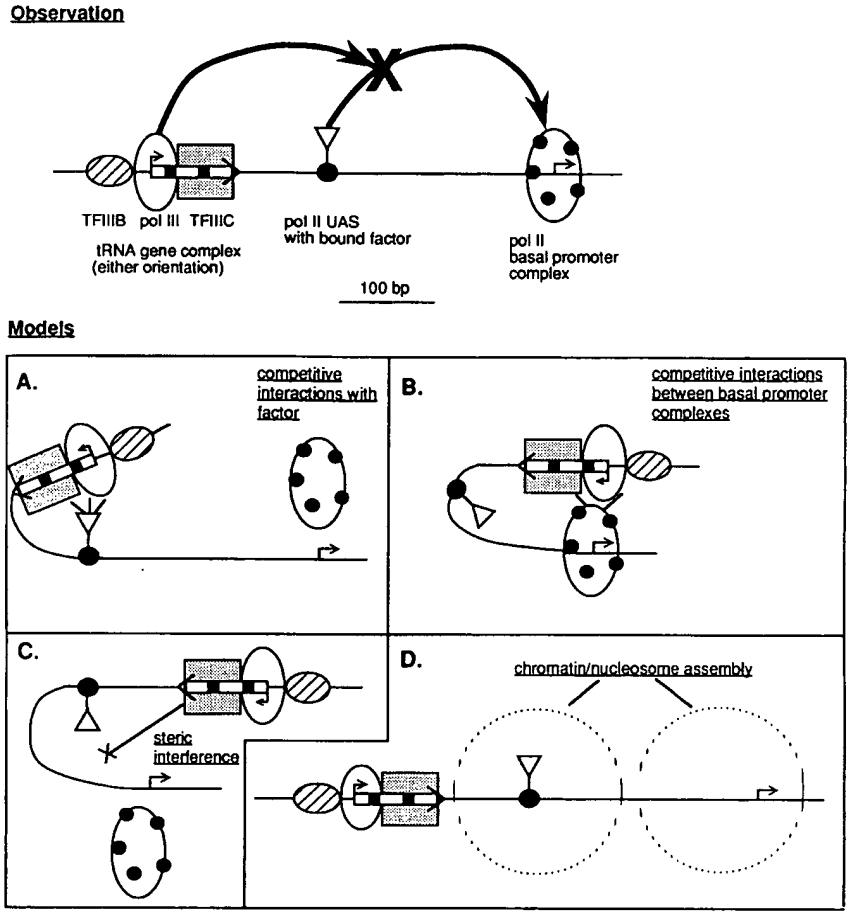

FIG. 7. Models for repression of adjacent pol II transcription by tRNA genes. Repression of pol II promoter activation is represented schematically at the top, with the tRNA gene complexes (in either orientation) interfering with activation by bound Gal4 protein. Four possible models for this repression are presented for discussion. In model A, components of the pol III complex interact with activation domains in the UAS complex to give competitive inhibition. In model B, components of the pol III complex interact with components of the pol II basal promoter machinery to block productive interactions with the UAS activation domains. In model C, the bulky pol III complex sterically blocks the ability of pol II and its basal transcription factors from interacting simultaneously with the UAS and the basal promoter. In model D, the pol III transcription complex attracts some type of repressor or repressing phased nucleosomes that block access by pol II transcription components.

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REFERENCES

1. **Andreadis, A., Y.-P. Hsu, G. B. Kohlhaw, and P. Schimmel.** 1982. Nucleotide sequence of yeast *LEU2* shows 5'-noncoding region has sequences cognate to leucine. Cell 31:319–325.
2. **Aparicio, O. S., B. L. Billington, and D. E. Gottschling.** 1991. Modifiers of position effect are shared between telomeric and silent mating-type loci in *S. cerevisiae*. Cell 66:1279–1287.
3. **Bartholomew, B., G. A. Kassavetis, and E. P. Geiduschek.** 1991. Two components of *Saccharomyces cerevisiae* transcription factor IIIB (TFIIIB) are stereospecifically located upstream of a tRNA gene and interact with the second-largest subunit of TFIIIC. Mol. Cell. Biol. 11:5181–5189.
4. **Bennetzen, J., and B. D. Hall.** 1982. The primary structure of the *Saccharomyces cerevisiae* gene for alcohol dehydrogenase I. J. Biol. Chem. 257:3018–3025.
5. **Boeke, J. D., and S. B. Sandmeyer.** 1991. Yeast transposable elements, p. 193–261. *In* J. R. Broach, E. W. Jones, and J. Pringle (ed.), The molecular and cellular biology of the yeast *Saccharomyces*: genome dynamic, protein synthesis, and energetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
6. **Brand, A. H., L. Breeden, J. Abraham, R. Sternglanz, and K. Nasmyth.** 1985. Characterization of a "silencer" in yeast: a DNA sequence with properties opposite to those of a transcriptional enhancer. Cell 41:41–48.

7. **Buratowski, S., and H. Zhou.** 1992. A suppressor of TBP mutations encodes an RNA polymerase III transcription factor with homology to TFIIB. Cell **71**:221–230.
8. **Burnol, A.-F., F. Margottin, J. Huet, G. Almouzni, M.-N. Prioleau, M. Méchali, and A. Sentenac.** 1993. TFIIIC relieves repression of U6 snRNA transcription by chromatin. Nature (London) **362**:475–477.
9. **Campbell, F. E., and D. R. Setzer.** 1992. Transcription termination by RNA polymerase III: uncoupling of polymerase release from termination signal recognition. Mol. Cell. Biol. **12**:2260–2272.
10. **Carey, M. F., K. Singh, M. Botchan, and N. R. Cozzarelli.** 1986. Induction of specific transcription by RNA polymerase III in transformed cells. Mol. Cell. Biol. **6**:3068–3076.
11. **Carlson, D. P., and J. Ross.** 1983. Human β-globin promoter and coding sequences transcribed by RNA polymerase III. Cell **34**:857–864.
12. **Carlson, D. P., and J. Ross.** 1986. Point mutation associated with hereditary persistence of fetal hemoglobin decreases RNA polymerase III transcription upstream of the affected γ-globin gene. Mol. Cell. Biol. **6**:3278–3282.
13. **Chalker, D. L., and S. B. Sandmeyer.** 1990. Transfer RNA genes are genomic targets for de novo transposition of the yeast retrotransposon Ty3. Genetics **126**:837–850.
14. **Chalker, D. L., and S. B. Sandmeyer.** 1991. Ty3 integrates within the region of RNA polymerase III transcription initiation. Genes Dev. **6**:117–128.
15. **Chung, J., D. J. Sussman, R. Zeller, and P. Leder.** 1987. The c-*myc* gene encodes superimposed RNA polymerase II and III promoters. Cell **51**:1001–1008.
16. **Clark, D. J., V. W. Bilanchone, L. J. Haywood, S. L. Dildine, and S. B. Sandmeyer.** 1988. A yeast sigma composite element, Ty3, has properties of a retrotransposon. J. Biol. Chem. **263**:1413–1423.
17. **Colbert, T., and S. Hahn.** 1992. A yeast TFIIB-related factor involved in RNA polymerase III transcription. Genes Dev. **6**:1940–1949.
18. **Cullen, B. R., P. T. Lomedico, and G. Ju.** 1984. Transcriptional interference in avian retroviruses—implications for the promoter insertion model of leukaemogenesis. Nature (London) **307**:241–246.
19. **Datta, S., C.-J. Soong, D. M. Wang, and M. L. Harter.** 1991. A purified adenovirus 289-amino-acid E1A protein activates RNA polymerase III transcription in vitro and alters transcription factor TFIIIC. J. Virol. **65**:5297–5304.
20. **DeLotto, R., and P. Schedl.** 1984. Internal promoter elements of transfer RNA genes are preferentially exposed in chromatin. J. Mol. Biol. **179**:607–628.
21. **Dieci, G., L. Duimio, F. Coda-Zabetta, K. U. Sprague, and S. Ottonello.** 1993. A novel RNA polymerase III transcription factor fraction that is not required for template commitment. J. Biol. Chem. **268**:11199–11207.
22. **Dingermann, T., U. Frank-Stoll, H. Werner, A. Wissmann, W. Hillen, M. Jacquet, and R. Marschalek.** 1992. RNA polymerase III catalyzed transcription can be regulated in *Saccharomyces cerevisiae* by the bacterial tetracycline repressor-operator system. EMBO J. **11**:1487–1492.
23. **Dingermann, T., H. Werner, A. Schütz, I. Zündorf, K. Nerke, D. Knecht, and R. Marschalek.** 1992. Establishment of a system for conditional gene expression using an inducible tRNA suppressor gene. Mol. Cell. Biol. **12**:4038–4045.
24. **Engelke, D. R., and J. M. Gottesfeld.** 1990. Chromosomal footprinting of transcriptionally active and inactive oocyte-type 5S rRNA genes of *Xenopus laevis*. Nucleic Acids Res. **18**:6031–6037.
25. **Evans, C. F., D. R. Engelke, and D. J. Thiele.** 1990. ACE1 transcription factor produced in *Escherichia coli* binds multiple regions within yeast metallothionein upstream activation sequences. Mol. Cell. Biol. **10**:426–429.
26. **Fascher, K.-D., J. Schmitz, and W. Hörz.** 1990. Role of *trans*-activating proteins in the generation of active chromatin at the *PHO5* promoter in *S. cerevisiae*. EMBO J. **9**:2523–2528.
27. **Fornace, A. J., and J. B. Mitchell.** 1986. Induction of B2 RNA polymerase III transcription by heat shock: enrichment for heat shock induced sequences in rodent cells by hybridization subtraction. Nucleic Acids Res. **14**:5793–5811.
28. **Goodman, H. M., M. V. Olson, and B. D. Hall.** 1977. Nucleotide sequence of a mutant eukaryotic gene: the yeast tyrosine-inserting ochre suppressor SUP4-o. Proc. Nat. Acad. Sci. USA **74**:5453–5457.
29. **Gottschling, D. E., O. M. Aparicio, B. L. Billington, and V. A. Zakian.** 1990. Position effect at *S. cerevisiae* telomeres. Cell **63**:751–762.
30. **Guarente, L.** 1983. Yeast promoters and *lacZ* fusions designed to study expression of cloned genes in yeast. Methods Enzymol. **101**:181–191.
31. **Gudenus, R., S. Mariotte, A. Moenne, A. Ruet, S. Memet, J.-M. Buhler, A. Sentenac, and P. Thuriaux.** 1988. Conditional mutants of *RPC160*, the gene encoding the largest subunit of RNA polymerase C in *Saccharomyces cerevisiae*. Genetics **119**:517–526.
32. **Guthrie, C., and J. Abelson.** 1982. Organization and expression of tRNA genes in *Saccharomyces cerevisiae*, p. 487–528. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*: metabolism and gene expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. **Han, M., and M. Grunstein.** 1988. Nucleosome loss activates yeast downstream promoters in vivo. Cell **55**:1137–1145.
34. **Hansen, L. J., and S. B. Sandmeyer.** 1990. Characterization of a transpositionally active Ty3 element and identification of the Ty3 integrase protein. J. Virol. **64**:2599–2607.
35. **Hannig, E. M., D. J. Thiele, and M. J. Leibowitz.** 1984. *Saccharomyces cerevisiae* killer virus transcripts contain template-coded polyadenylate tracts. Mol. Cell. Biol. **4**:101–109.
36. **Hauber, J., R. Stucka, R. Krieg, and H. Feldmann.** 1988. Analysis of yeast chromosomal regions carrying members of the glutamate tRNA gene family: various transposable elements are associated with them. Nucleic Acids Res. **16**:10623–10634.
37. **Herskowitz, I.** 1989. A regulatory hierarchy for cell specialization in yeast. Nature (London) **342**:749–757.
38. **Hirschhorn, J. N., S. A. Brown, C. D. Clark, and F. Winston.** 1992. Evidence that SNF2/SW12 and SNF5 activate transcription in yeast by altering chromatin structure. Genes Dev. **6**:2288–2298.
39. **Hirschman, J. E., K. J. Durbin, and F. Winston.** 1988. Genetic evidence for promoter competition in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8**:4608–4615.
40. **Huet, J., and A. Sentenac.** 1992. The TATA-binding protein participates in TFIIIB assembly on tRNA genes. Nucleic Acids Res. **20**:6451–6454.
41. **Huibregtse, J. M., and D. R. Engelke.** 1989. Genomic footprinting of a yeast tRNA gene reveals stable complexes over the 5′ flanking region. Mol. Cell. Biol. **9**:2195–2205.
42. **Huibregtse, J. M., D. R. Engelke, and D. J. Thiele.** 1989. Copper-induced binding of cellular factors to yeast metallothionein upstream activation sequences. Proc. Natl. Acad. Sci. USA **86**:65–69.
43. **Huibregtse, J. M., C. F. Evans, and D. R. Engelke.** 1987. Comparison of tRNA gene transcription complexes formed *in vitro* and in nuclei. Mol. Cell. Biol. **7**:3212–3220.
44. **Huibregtse, J. M., P. D. Good, G. T. Marczynski, J. A. Jaehning, and D. R. Engelke.** 1993. Gal4 protein binding is required, but not sufficient for transcriptional regulation. J. Biol. Chem. **268**:22219–22222.
45. **Hull, M. W., G. Thomas, J. M. Huibregtse, and D. R. Engelke.** 1991. Protein-DNA interactions in vivo—examining genes in *Saccharomyces cerevisiae* and *Drosophila melanogaster* by chromatin footprinting. Methods Cell Biol. **35**:383–415.
46. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
47. **Ji, H., D. P. Moore, M. A. Blomberg, L. T. Braiterman, D. F. Voytas, G. Natsoulis, and J. D. Boeke.** 1993. Hotspots for unselected Ty1 transposition events on yeast chromosome III

are near tRNA genes and LTR sequences. Cell 73:1007–1018.
48. **Johnson, D. L., R. S. Fan, and M. L. Treinies.** 1991. Analysis of the molecular mechanisms for the species-specific transcription of *Drosophila* and human tRNA gene transcription components. J. Biol. Chem. 266:16037–16043.
49. **Johnston, M., and R. W. Davis.** 1984. Sequences that regulate the divergent *GAL1-GAL10* promoter in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 4:1440–1448.
50. **Kassavetis, G. A., B. Bartholomew, J. A. Blanco, T. E. Johnson, and E. P. Geiduschek.** 1991. Two essential components of the *Saccharomyces cerevisiae* transcription factor TFIIIB: transcription and DNA-binding properties. Proc. Natl. Acad. Sci. USA 88:7308–7312.
51. **Kassavetis, G. A., J. A. Blanco, T. E. Johnson, and E. P. Geiduschek.** 1992. Formation of open and elongating transcription complexes by RNA polymerase III. J. Mol. Biol. 226:47–58.
52. **Kassavetis, G. A., B. R. Braun, L. H. Nguyen, and E. P. Geiduschek.** 1990. *S. cerevisiae* TFIIIB is the transcription initiation factor proper of RNA polymerase III, while TFIIIA and TFIIIC are assembly factors. Cell 60:235–245.
53. **Kassavetis, G. A., C. A. P. Joazeiro, M. Pisano, E. P. Geiduschek, T. Colbert, S. Hahn, and J. A. Blanco.** 1992. The role of the TATA-binding protein in the assembly of the multisubunit yeast RNA polymerase III transcription factor, TFIIIB. Cell 71:1055–1064.
54. **Kassavetis, G. A., D. L. Riggs, R. Negri, L. H. Nguyen, and E. P. Geiduschek.** 1989. Transcription factor IIIB generates extended DNA interactions in RNA polymerase III transcription complexes on tRNA genes. Mol. Cell. Biol. 9:2551–2566.
55. **Kayne, P. S., U.-J. Kim, M. Han, J. R. Mullen, F. Yoshizaki, and M. Grunstein.** 1988. Extremely conserved histone H4 N terminus is dispensable for growth but essential for repressing the silent mating loci in yeast. Cell 55:27–39.
56. **Keleher, C. A., M. J. Redd, J. Schultz, M. Carlson, and A. D. Johnson.** 1992. Ssn6-Tup1 is a general repressor of transcription in yeast. Cell 68:709–719.
57. **Kinsey, P. T., and S. B. Sandmeyer.** 1991. Adjacent pol II and pol III promoters: transcription of the yeast retrotransposon Ty3 and a target tRNA gene. Nucleic Acids. Res. 19:1317–1324.
58. **Kiss, T., C. Marshallsay, and W. Filipowicz.** 1991. Alteration of the RNA polymerase specificity of U3 snRNA genes during evolution and in vitro. Cell 65:517–526.
59. **Kohrer, K., and H. Domdey.** 1991. Preparation of high molecular weight RNA. Methods Enzymol. 194:398–405.
60. **Kurjan, J., and B. D. Hall.** 1980. Mutations at the yeast *SUP4* $tRNA^{Tyr}$ locus: DNA sequence changes in mutants lacking suppressor activity. Cell 20:701–709.
61. **Lania, L., A. Pannuti, G. La Mantia, and C. Basilico.** 1987. The transcription of B2 repeated sequences is regulated during the transition from quiescent to proliferative state in cultured rodent cells. FEBS Lett. 219:400–404.
62. **Lee, J.-Y., C. F. Evans, and D. R. Engelke.** 1991. Expression of RNase P RNA in *Saccharomyces cerevisiae* is controlled by an unusual RNA polymerase III promoter. Proc. Natl. Acad. Sci. USA 88:6986–6990.
63. **Léveillard, T., G. A. Kassavetis, and E. P. Geiduschek.** 1991. *Saccharomyces cerevisiae* transcription factors IIIB and IIIC bend the DNA of a $tRNA^{Gln}$ gene. J. Biol. Chem. 266:5162–5168.
64. **Levine, M., and J. L. Manley.** 1989. Transcription repression of eukaryotic promoters. Cell 59:405–408.
65. **Lobo, S. M., J. Lister, M. L. Sullivan, and N. Hernandez.** 1991. The cloned RNA polymerase II transcription factor IID selects RNA polymerase III to transcribe the human U6 gene *in vitro*. Genes Dev. 5:1477–1489.
66. **Lobo, S. M., M. Tanaka, M. L. Sullivan, and N. Hernandez.** 1992. A TBP complex essential for transcription from TATA-less but not TATA-containing RNA polymerase III promoters is part of the TFIIIB fraction. Cell 71:1029–1040.
67. **López-De-León, A., M. Librizzi, K. Puglia, and I. M. Willis.** 1992. *PCF4* encodes an RNA polymerase III transcription factor with homology to TFIIB. Cell 71:211–220.
68. **Lorch, Y., and R. D. Kornberg.** 1985. A region flanking the *GAL7* gene and a binding site for *GAL4* protein as upstream activating sequences in yeast. J. Mol. Biol. 186:821–824.
69. **Marschalek, R., T. Brechner, E. Amon-Böhm, and T. Dingermann.** 1989. Transfer RNA genes: landmarks for integration of mobile genetic elements in *Dictyostelium discoideum*. Science 244:1493–1496.
70. **Marschalek, R., J. Hofmann, G. Schumann, R. Gösseringer, and T. Dingermann.** 1992. Structure of DRE, a retrotransposable element which integrates with position upstream of *Dictyostelium discoideum* tRNA genes. Mol. Cell. Biol. 12:229–239.
71. **Murphy, S., J.-B. Yoon, T. Gerster, and R. G. Roeder.** 1992. Oct-1 and Oct-2 potentiate functional interactions of a transcription factor with the proximal sequence element of small nuclear RNA genes. Mol. Cell. Biol. 12:3247–3261.
72. **Nelbock, P., R. Stucka, and H. Feldmann.** 1985. Different patterns of transposable elements in the vicinity of tRNA genes in yeast: a possible clue to transcriptional modulation. Biol. Chem. Hoppe-Seyler 366:1041–1051.
73. **Newman, A. J., R. C. Ogden, and J. Abelson.** 1983. tRNA gene transcription in yeast: effects of specified base substitutions in the intragenic promoter. Cell 35:117–125.
74. **Okada, N.** 1991. SINEs. Curr. Opin. Genet. Dev. 1:498–504.
75. **Rogers, J. H.** 1985. The origin and evolution of retroposons. Int. Rev. Cytology 93:187–279.
76. **Roth, S. Y., A. Dean, and R. T. Simpson.** 1990. Yeast α2 repressor positions nucleosomes in TRP1/ARS1 chromatin. Mol. Cell. Biol. 10:2247–2260.
77. **Roth, S. Y., M. Shimizu, L. Johnson, M. Grunstein, and R. T. Simpson.** 1992. Stable nucleosome positioning and complete repression by the yeast α2 repressor are disrupted by amino-terminal mutations in histone H4. Genes Dev. 6:411–425.
78. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
79. **Sandmeyer, S. B., V. W. Bilanchone, D. J. Clark, P. Morcos, G. F. Carle, and G. M. Brodeur.** 1988. Sigma elements are position-specific for many different yeast tRNA genes. Nucleic Acids Res. 16:1499–1515.
80. **Sandmeyer, S. B., and M. V. Olson.** 1982. Insertion of a repetitive element at the same position in the 5′ flanking regions of two dissimilar tRNA genes. Proc. Natl. Acad. Sci. USA 79:7674–7678.
81. **Schmid, A., K.-D. Fascher, and W. Hörz.** 1992. Nucleosome disruption at the yeast *PHO5* promoter upon *PHO5* induction occurs in the absence of DNA replication. Cell 71:853–864.
82. **Schnell, R., and J. Rine.** 1986. A position effect on the expression of a tRNA gene mediated by the *SIR* genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 6:494–501.
83. **Shaw, K. J., and M. V. Olson.** 1984. Effects of altered 5′-flanking sequences on the in vivo expression of a *Saccharomyces cerevisiae* $tRNA^{Tyr}$ gene. Mol. Cell. Biol. 4:657–665.
84. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
85. **Shimizu, M., S. Y. Roth, C. Szent-Gyorgyi, and R. T. Simpson.** 1991. Nucleosomes are positioned with base pair precision adjacent to the α2 operator in *Saccharomyces cerevisiae*. EMBO J. 10:3033–3041.
86. **Simmen, K. A., J. Bernués, H. D. Parry, H. G. Stunnenberg, A. Berkenstam, B. Cavallini, J.-M. Egly, and I. W. Mattaj.** 1991. TFIID is required for in vitro transcription of the human U6 gene by RNA polymerase III. EMBO J. 10:1853–1862.
87. **Simpson, R. T.** 1990. Nucleosome positioning can affect the function of a *cis*-acting DNA element in vivo. Nature (London) 343:387–389.
88. **Straka, C., and W. Hörz.** 1991. A functional role for nucleosomes in the repression of a yeast promoter. EMBO J. 10:361–368.
89. **Sussman, D. J., J. Chung, and P. Leder.** 1991. In vitro and in

vivo analysis of the c-*myc* RNA polymerase III promoter. Nucleic Acids Res. **19**:5045–5052.
90. **Sutcliffe, J. G., R. J. Milner, J. M. Gottesfeld, and R. A. Lerner.** 1984. Identifier sequences are transcribed specifically in brain. Nature (London) **305**:237–241.
91. **Swanson, R. N., C. Conesa, O. Lefebvre, C. Carles, A. Ruet, E. Quemeneur, J. Gagnon, and A. Sentenac.** 1991. Isolation of *TFC1*, a gene encoding one of two DNA-binding subunits of yeast transcription factor τ (TFIIIC). Proc. Natl. Acad. Sci. USA **88**:4887–4891.
92. **Taggart, A. K. P., T. S. Fisher, and B. F. Pugh.** 1992. The TATA-binding protein and associated factors are components of pol III transcription factor TFIIIB. Cell **71**:1015–1028.
93. **Takahashi, K., S. Murakami, Y. Chikashige, O. Niwa, and M. Yanagida.** 1991. A large number of tRNA genes are symmetrically located in fission yeast centromeres. J. Mol. Biol. **218**:13–17.
94. **Thiele, D. J.** 1988. *ACE1* regulates expression of the *Saccharomyces cerevisiae* metallothionein gene. Mol. Cell. Biol. **8**:2745–2752.
95. **Tiedge, H., R. T. Fremeau, P. H. Weinstock, O. Arancio, and J. Brosius.** 1991. Dendritic location of neural BC1 RNA. Proc. Natl. Acad. Sci. USA **88**:2093–2097.
96. **Tomilin, N. V., S. M. M. Iguchi-Ariga, and H. Ariga.** 1990. Transcription and replication silencer element is present within conserved region of human Alu repeats interacting with nuclear protein. FEBS Lett. **263**:69–72.
97. **Vasseur, M., H. Condamine, and P. Deprep.** 1985. RNAs containing B2 repeated sequences are transcribed in the early stages of mouse embryogenesis. EMBO J. **4**:1749–1753.
98. **Venegas, A., M. Quiroga, J. Zaldivar, W. J. Rutter, and P. Valenzuela.** 1979. Isolation of yeast $tRNA^{Leu}$ genes. DNA sequence of a cloned $tRNA^{Leu}_3$ gene. J. Biol. Chem. **254**: 12306–12309.
99. **Waldschmidt, R., and K. H. Seifart.** 1992. TFIIA is required for in vitro transcription of mammalian U6 genes by RNA polymerase III. J. Biol. Chem. **267**:16359–16364.
100. **Waldschmidt, R., I. Wanandi, and K. H. Seifart.** 1991. Identification of transcription factors required for the expression of mammalian U6 genes in vitro. EMBO J. **10**:2595–2603.
101. **Watson, J. B., and J. G. Sutcliff.** 1987. Primate brain-specific cytoplasmic transcript of the Alu repeat family. Mol. Cell. Biol. **7**:3324–3327.
102. **Werner, M., S. Hermann-Le Denmat, I. Treich, A. Sentenac, and P. Thuriaux.** 1992. Effect of mutations is a zinc-binding domain of yeast RNA polymerase C (III) on enzyme function and subunit association. Mol. Cell. Biol. **12**:1087–1095.
103. **White, R. J., and S. P. Jackson.** 1992. Mechanism of TATA-binding protein to a TATA-less class III promoter. Cell **71**: 1041–1053.
104. **Willis, I., A. Oksman, and A. López-De-León.** 1992. The PCF1-1 mutation increases the activity of the transcription factor (TF) IIIB fraction from *Saccharomyces cerevisiae*. Nucleic Acids Res. **20**:3725–3730.
105. **Young, L. S., D. H. Rivier, and K. U. Sprague.** 1991. Sequences far downstream from the classical tRNA promoter elements bind RNA polymerase III transcription factors. Mol. Cell. Biol. **11**:1382–1392.